REMARKS

A check for $120 for the fee for a one-month extension of time accompanies this response. Any fees that may be due in connection with the filing of this paper or with this application may be charged to Deposit Account No. 06-1050. If a Petition for extension of time is needed, this paper is to be considered such Petition. Supporting art accompanies this response.

Claims 1-9, 11-31, 37-40, 46, 49-51, 56-72, 75-77 and 108 are pending. Claims 10, 41, 42 and 45 are cancelled herein without prejudice or disclaimer. Applicant reserves the right to file a continuation application directed to cancelled subject matter. Claims 1, 9, 29-31, 49-51, 58, 63, 71, 72, 76 and 77 are amended herein to more distinctly claim the subject matter. Claims 1 and 58 are amended to define the substituents of the optionally substituted groups. Basis for the amendment is found throughout the specification (*e.g.*, see page 11, line 26 through page 12, line 9). Claims 1 and 58 also are amended to separate the substituents for variables X and Z. Claims 1, 9, 29-31, 49, 50, 58, 63, 71 and 72 are amended to cancel subject matter directed to substituents that when taken together form a carbocyclic or heterocyclic ring. Applicant reserves the right to file a continuation application directed to cancelled subject matter. Claims 51 and 76 are amended to more distinctly claim the substituents for X and Z. Basis for new claim 108 is found throughout the specification (for example, see pages 3-7 and original claim 1). No new matter is added.

THE REJECTION OF CLAIMS 1-31, 37-42, 45, 46, 49-51, 58-72 AND 75-77 UNDER 35 U.S.C. § 112, FIRST PARAGRAPH – Scope of Enablement

Claims 1-31, 37-42, 45, 46, 49-51, 58-72 and 75-77 are rejected under 35 U.S.C. § 112, first paragraph as allegedly containing subject matter not described in the specification in such a way as to enable one of skill in the art to make and/or use the claimed subject matter. The Examiner states that the compound and pharmaceutical composition claims are only enabled in part because the instant claims include terms that allegedly are incompletely defined. The Examiner also alleges that it would require undue experimentation to practice the full scope of the claims. The Examiner alleges that "the scope is excessive in view of the disclosed exemplifications." Applicant respectfully traverses the rejection.

RELEVANT LAW

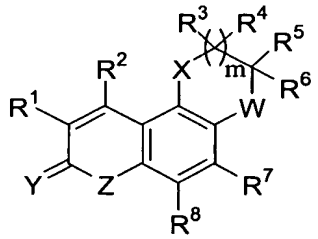
The test of enablement is whether one skilled in the art can make and use what is claimed based upon the disclosure in the application and information known to those of skill in the art without undue experimentation. *United States v. Telectronics, Inc.*, 8 USPQ2d

1217 (Fed. Cir. 1988). A certain amount of experimentation is permissible as long as it is not undue. A patent application need not teach, and preferably omits, what is well known in the art. *Spectra-Physics, Inc. v. Coherent , Inc.*, 3 USPQ2d 1737 (Fed. Cir. 1987). Indeed, "not everything necessary to practice the invention need be disclosed. In fact, what is well-known is best omitted." *In re Buchner*, 929 F.2d 660, 661, 18 U.S.P.Q.2d 1331, 1332. Showing every combination of substituents is unnecessary.

A considerable amount of experimentation is permissible, particularly if it is routine experimentation. The amount of experimentation that is permissible depends upon a number of factors, which include: the quantity of experimentation necessary, the amount of direction or guidance presented, the presence or absence of working examples, the nature of the invention, the state of the prior art, the relative skill of those in the art, the predictability of the art, and the breadth of the claims. *See, Ex parte Forman*, 230 USPQ 546 (Bd. Pat. App. & Int'f 1986); see also *In re Wands*, 8 USPQ2d 1400 (Fed. Cir. 1988).

THE CLAIMS

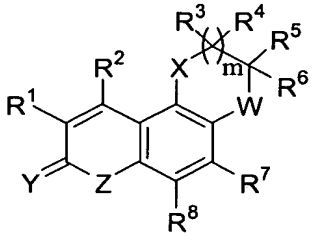
Claim 1 is directed to a compound having the formula:



(I)

where the substituents are as recited in the claims. Claims 2-9, 11-31, 37-40, 46 and 49-51 ultimately depend from claim 1 and are directed to various embodiments thereof.

Claim 58 is directed to a pharmaceutical composition that includes a pharmaceutically acceptable carrier and a compound of formula:



(I)

where the substituents are as recited in the claims. Claims 59-72, 75 and 76 ultimately depend from claim 58 and are directed to various embodiments thereof.

ANALYSIS

Applicant respectfully submits that the Examiner rejects claims 1-31, 37-42, 45, 46, 49-51, 58-72 and 75-77 under 35 U.S.C. § 112, first paragraph on page 2 and on page 3 of the Office Action. Both rejections state that the claims are rejected because it is alleged that practicing the full scope of the claims requires undue experimentation. Applicant respectfully requests that the Examiner clarify the difference between the rejection on page 2 and the rejection on page 3 of the Office Action so that Applicant can address the rejections with particularity. In order to be fully responsive, Applicant provides the following traverse.

The *In re Wands* factors

Applying the *In re Wands* factors to the instant facts reveals that the amount of experimentation is not undue. The analysis and arguments set forth in the previous responses of record are incorporated by reference herein.

a. The scope of the claims.

The pending claims recite compounds of Formula I and compositions thereof. The Examiner alleges that:

> The repeated use of the term "may be optionally substituted" without specifying the substituents implied thereby renders the breadth of the claim excessive because said term implies that the unnamed substituents is/are open to all possible alternatives.

First, the pending claims do not include the recitation "may be optionally substituted." The pending claims include the recitation "optionally substituted." Further, as discussed above, claims must be read in view of the specification. See *e.g.*, MPEP § 2106 ("An applicant is entitled to be his or her own lexicographer, and in many instances will provide an explicit definition for certain terms used in the claims. Where an explicit definition is provided by the applicant for a term, that definition will control interpretation of the term as it is used in the claim."); MPEP § 608.01(o) ("The meaning of every term used in any of the claims should be apparent from the descriptive portion of the specification"); MPEP § 2173.05 ("When the specification states the meaning that a term in the claims is intended to have, the claim is examined using that meaning in order to achieve a complete exploration of the applicant's invention and its relation to the prior art."). The term "optionally substituted" is expressly defined in the specification. Thus, the recitation "optionally substituted" does not imply "that the unnamed substituents is/are open to all possible alternatives" as alleged by the Examiner. Thus, reciting that definition in the claims is not necessary. Without a acquiescing to the Examiner's allegation and solely to expedite issuance of the application, independent claims 1

and 58 are amended herein to recite the substituents encompassed by the recitation "optionally substituted" in the claims.

b. Nature of the Invention

The specification provides a general description of non-steroidal compounds that are high-affinity, high-specificity agonists, partial agonists (*i.e.*, partial activators and/or tissue-specific activators) and antagonists for androgen receptors (AR). The claimed subject matter is directed to androgen receptor modulator compounds and pharmaceutical compositions containing such compounds.

The Examiner states that the nature of the invention "includes a method of testing, a method of purification and a vast number of medicinal treatment[s] wherein compounds of instant claim 1 are administered to a host in need of such treatment." Applicant respectfully submits that the pending claims are directed to compounds as recited in the claims and to pharmaceutical compositions thereof. There currently are no method claims pending.

c. State of the Prior Art

Applicant respectfully submits that, at the time of the priority application, the mechanism of action of the intracellular receptors and the effects of small molecule agonists, antagonist or partial agonists on IR-mediated transcription modulation was well known to the skilled artisan (for example, see Rosen *et al.* (*J. Med. Chem.*, 1995, vol. 38, No. 25, pp 4855-4874, a copy of which is provided herein). Androgen receptor agonist compounds and their use as therapeutic agents also was known to those skilled in the medical arts. For example, Rosen *et al.* provides an overview of diseases and conditions that share mediation by androgen receptors as an underlying etiology. For example, Rosen *et al.* recites on page 4862:

> "Androgens are synthesized in the testes, adrenal cortex, and ovaries. The net effect of endogenous androgens reflects the combined actions of the secreted hormone, testosterone; its 5α-reduced metabolite, dihydrotestosterone; and its estrogenic derivative, estradiol. Androgens serve different functions at different stages of male development and have clear therapeutic uses in the treatment of hypogonadism, growth retardation, breast carcinoma, and osteoporosis. The actions of androgens are mediated through AR."

Applicant also submits that at the time of the priority application, androgen receptor agonist compounds were either in clinical trials or were available to the public for the treatment of hypogonadism, metastatic breast cancer, anemias, as anabolic agents and for the treatment of other diseases or conditions. For example, Testoderm®, a testosterone transdermal patch, was approved in the U.S. in 1993 for hormone replacement therapy in hypogonadal men. Androderm®, also a transdermal testosterone patch, was approved in the

U.S. in 1995 for the treatment of hypogonadism. Testred®, which contains the AR agonist methyltestosterone, was approved in the U.S. in 1973 for hormone replacement therapy in hypogonadal men as well as for the treatment of metastatic breast cancer in women. Anadrol®-50, which contains the AR agonist oxymetholone, was approved in the U.S. in 1972 and is used in the treatment of anemias caused by deficient red cell production. It also is well known that testosterone, the native androgen and AR agonist, is an anabolic agent. Therefore, it was well known in the prior art at the time of filing the original application that androgen receptor agonist compounds and compositions that include AR agonist compounds are useful in male hormone replacement therapy, in stimulation of hematopoiesis, as anabolic agents, and in the treatment of wasting diseases, hypogonadism and breast cancer.

Applicant also respectfully submits that, at the time of filing the priority application, the use of androgen receptor antagonists as therapeutic agents was known to those skilled in the medical arts. For example, Singh *et al.*, teaches that androgen receptor antagonists are useful for treating prostate cancer, acne, seborrhea, hirsuitism and androgenic alopecia (Singh *et al.*, Current Medicianl Chemistry 7: 211-247 (2000)). Rosen *et al.* (*J. Med. Chem.*, 1995, vol. 38, No. 25, pp 4855-4874) also provides an overview of diseases and conditions that share an etiology of being mediated by androgen receptor antagonists. For example, Rosen *et al.* recites on page 4862:

> "Compounds that block the action or synthesis of androgens have proven useful in treatment of diseases such as prostate cancer, prostatic hypertrophy, hirsutism, male pattern baldness, and acne. Among the most potent orally active anti-androgens is cyproterone acetate. This compound possesses progestational activity and suppresses the secretion of gonadotrphins, both of which are unwanted side effects. Other anti-androgens include flutamide, a prodrug for the active metabolite, 2-hydroxyflutamide, casodex, and an analogue of nilutamide."

Applicant also submits that at the time of filing the instant application, several androgen receptor antagonist compounds were either in clinical trials or were available to the public for the treatment of the diseases or conditions listed above. For example, Eulexin®, which contains the androgen receptor antagonist flutamide, was approved in the U.S. in 1989 for the treatment of prostate cancer. Casodex® (bicalutamide), an androgen receptor antagonist, was approved in 1995 for the treatment of prostate cancer. The AR antagonist cyproterone acetate was approved for use in Europe as early as 1978 for the treatment of female acne and hirsutism (Gruber *et al.*, Arch Dermatol 134: 459-463 (1998), Venturoli *et al*, J Clin Endocrinology & Metabolism 84(4): 1304-1310 (1999)).

In addition, a number of methods and assays for identifying agonists, partial agonists or antagonists of the steroid receptors was known at the time of filing the original application. For example, Berger *et al.* teaches a co-transfection assay (Berger *et al.*, J. Steroid Biochem. Molec. Biol. 41: 773 (1992)). Berger *et al.* teaches that activity in the co-transfection assay correlates very well with known *in vivo* activity, such that the co-transfection assay functions as a qualitative and quantitative predictor of a tested compounds *in vivo* pharmacology.

Thus, at the time of filing of the instant application, a broad body of knowledge had amassed in the areas of pharmaceutical sciences, medicine, and biochemistry directed to compounds that agonize or antagonize the steroid receptors, including androgen receptors, and to the use of compounds that agonize or antagonize the steroid receptors, such as androgen receptors, for treatment of diseases and conditions.

d. Level of Skill in the Art

As the Examiner noted, the skill in the art of chemical synthesis is high. That skill, together with the instant specification, including cited and incorporated references, allow the skilled artisan to make any and all of the claimed compounds. The Examiner states that "the level of skill in the medicinal arts is moderate because it is unclear which if any of the compounds disclosed herein are active against one or more specific disease conditions." Applicants respectfully disagree. The level of skill in the medical arts is independent of the issues. The skill of the medical practitioner does not vary.

Applicant respectfully submits that the level of skill in the medical arts is high. This is evidenced by the art in this area, which is authored primarily by those with Ph.D. and M.D. degrees and is intended for an audience of similarly highly skilled individuals, primarily in the fields of biochemical, pharmaceutical, or medical arts. The numerous articles and patents made of record in this application, authored and reviewed by those known in the art, address a highly skilled audience, and further evidence the high level of skill in this art. Therefore, the amount of disclosure required to meet the enablement requirement is minimal.

With respect to the rejection, it is respectfully submitted that no evidence is provided to support the Examiner's position that "the level of skill in the medicinal arts is moderate because it is unclear which if any of the compounds disclosed herein are active against one or more specific disease conditions." The Examiner is reminded that MPEP 2144.03 states:

> The Examiner may take official notice of facts outside of the record which are capable of instant and unquestionable demonstration as being "well- known" in the art. *In re Ahlert*, 424 F.2d 1088, 1091, 165 USPQ 418, 420 (CCPA 1970). . . ..

The facts of which the Examiner is taking notice are conclusory and are not capable of instant and unquestionable demonstration as being "well-known" in the art. MPEP 2144.03 continues:

> If justified, the examiner should not be obliged to spend time to produce documentary proof. If the knowledge is of such notorious character that official notice can be taken, it is sufficient so to state. *In re Malcolm*, 129 F.2d 529, 54 USPQ 235 (CCPA 1942). If the applicant traverses such an assertion the examiner should cite a reference in support of his or her position.

If this position is maintained, the Examiner must provide a reference supporting this position.

e. Predictability of the Art

The Examiner alleges that the level of predictability in the art is indeterminate because it allegedly is not clear which compounds are active as androgen receptor agonists or antagonists. Applicant respectfully disagrees.

The instant application provides detailed teachings of *in vitro* and *in vivo* assays that allow one of skill in the art to test the compounds as instantly claimed for androgen receptor activity. The instant application, for example pages 110-114, provides a highly detailed teaching of *in vitro* assays, such as the "cis-trans" or "co-transfection" assay. Table 1 on page 115 of the specification provides *in vitro* binding data of exemplary compounds disclosed in the instant application that exhibit androgen receptor agonist activity, partial agonist activity, or antagonist activity. These assays are known in the art (see Evans *et al.*, Science 240: 889-95 (1988) and correlate well with *in vivo* activity and can function as a qualitative and quantitative predictor of a tested compounds *in vivo* pharmacology (Berger *et al.*, J. Steroid Biochem. Molec. Biol. 41: 773 (1992)). The data indicates that all of the exemplary compounds possess androgen receptor agonist, partial agonist or antagonist activity.

The compounds of the instant application are further characterized for their specificity for the androgen receptor by examining the *in vitro* binding activity with other members of the steroid receptors. Table 2 on page 116 provides binding data for exemplary compounds disclosed in the instant application with the androgen, progesterone, estrogen, glucocorticoid and mineralcorticoid receptors. As noted above, the androgen receptor agonist activity or antagonist activity shown in the *in vitro* assays correlates very well with *in vivo* activity. Once the androgen receptor activity of the compounds have been established, the application of those compounds to the treatment of any diseases responsive to androgen receptor agonists or antagonists, as discussed above, is well within the routine skills of those skilled in the art through reference to the present specification as well as the general and specialized knowledge of those working in this recognized field. Further, formulating such compounds into a

pharmaceutical composition and administration of such compositions to a subject is well known in the medical arts.

f. The amount of direction or guidance presented and the presence of working examples

The Examiner admits that the specification discloses 150 exemplary compounds that are precursors, intermediates or final products of the claimed compounds. The specification also teaches seven generic synthesis schemes (for example, see page 33, 35, 37, 38, 39, 41 and 42). The application names over 150 exemplary AR modulator compounds (for example, see page 29 through 32 and claims 56 and 57). The specification also provides over 50 working examples and two screening assays. It is respectfully submitted that the direction provided by the specification is sufficient to allow one of skill in the art to synthesize, test and administer any and all compounds of the claimed subject matter. Modification of the reaction conditions or choice of starting materials is routine and within the scope of the teachings in the application and knowledge of one of skill in the art.

For example, Scheme I outlines the synthesis of 5-hydroxy-6-bromo-quinoline compounds **6** starting from a phenylenediamine derivative, for example, 5-chloro-1,3-phenylenediamine. Other phenylenediamine derivatives also can be used in the synthetic sequence outlined in Scheme I. For example, use of 5-chloro-1,4-phenylenediamine as the starting material in Scheme I results in the synthesis of 6-hydroxy-5-bromo-quinoline compounds.

Schemes III and V outline the synthesis of 7-nitro-1,4-benzoxazine compounds, which begin with the chemo- and regioselective N-alkylation of an amino alcohol onto a 3,4-dihalonitrobenzene, such as, for example, 3,4-difluoronitrobenzene. One of skill in the art would recognize that selective protection of the nitrogen atom of the aminoalcohol **13**, with, for example, di-tert-butyl dicarbonate, prior to reaction with 3,4-difluoronitrobenzene would result in the reaction of the alcohol moiety of the aminoalcohol at the 4-position of 3,4-difluoronitrobenzene. Removal of the protecting group on the nitrogen, with, for example, acid, followed by treatment with base would provide 6-nitro-1,4-benzoxazine compounds, which are regioisomers of structures **15** and **24**.

Scheme VI outlines a racemic route to 7-nitro-1,4-benzoxazine compounds **24** that begins with the *N*-alkylation of a 2-amino-5-nitrophenol by treatment with an aldehyde, its corresponding hydrate or hemiacetal, in the presence of a reducing agent, for example, sodium cyanoborohydride. However, use of a 2-amino-4-nitrophenol derivative as the starting material, instead of 2-amino-5-nitrophenol as depicted in Scheme VI, would provide 6-nitro-

1,4-benzoxazine compounds. 6-Nitro-1,4-benzoxazine compounds are regioisomers of compounds of structure **24**. One of skill in the art can readily follow these schemes or known variations of such schemes with any of a vast number of commonly available starting materials to arrive at the claimed subject matter. The art of chemical synthesis is predictable and is dictated by recognized chemical reactions and constraints. There is nothing of record to suggest that production or use of any of the claimed compounds or compositions would require development of new procedures or excessive experimentation. Organic synthesis methods have been used for decades. Hence, any experimentation would be routine to the skilled artisan. Therefore, in view of the teachings of the specification, in combination with what was known at the time the priority application was filed, Applicant respectfully submits that the claimed compounds can be prepared predictably using the methods disclosed in the specification or that are known to those skilled in this art.

Similarly, one of skill in the art can assess the activity of any of the claimed compounds using the binding assay or the co-transfection assay, both of which are disclosed in the specification, though one of skill in the art can assess compounds using other known assays. Various screening assays for assessing the ability of a compound or composition to modulate the transcriptional ability of intracellular receptors are known to those of skill in the art, such as those described in U.S. Pat. Nos. 4,981,784, 5,071,773, 5,298,429, and 5,506,102 and in WO89/05355, WO91/06677, WO92/05447, WO93/11235, WO93/23431, WO94/23068, WO95/18380 and CA 2,034,220. Further, formulating such compounds into a pharmaceutical composition and administration of such compositions to a subject is well know in the medical arts. Thus, preparation and administration of pharmaceutical compounds also is predictable. Finally, administration of compounds is routine to one of skill in the medical arts. Thus, it is respectfully submitted that the direction provided by the specification is sufficient to allow one of skill in the art to synthesize, test and administer any and all compounds of the claimed subject matter. Therefore, Applicant respectfully submits that one skilled in the art can make and use what is claimed based upon the disclosure in the application and information known to those of skill in the art without undue experimentation.

g. The amount of experimentation required

There is nothing of record to suggest that production or use of any of the claimed compounds or compositions would require development of new procedures or excessive experimentation. Organic synthesis methods have been used for decades. As discussed above, bioassays for evaluating whether compounds are functional ligands for receptor

proteins were known in the art since at least 1991. Such assays are routine in this art and do not require excessive experimentation. Applicant notes that "a considerable amount of experimentation is permissible, if it is merely routine . . ." *In re Wands* 858 F.3d 731, 737 (Fed Cir. 1988).

CONCLUSION

In light of the scope of the claims, the nature of the claimed subject matter, the state of the prior art, the high level of skill of those in this art, the predictability of the art, the amount of direction and guidance presented in the specification, the presence of over 50 working examples, the low amount of experimentation required and the fact that any required experimentation is routine, Applicant respectfully submits that it would not require undue experimentation for a person skilled in the art to make and use the claimed compounds and compositions. Therefore, the specification is enabling for making and using the full scope of the claimed subject matter. Applicant respectfully requests that the rejection be reconsidered and withdrawn.

REBUTTAL TO THE EXAMINER'S ARGUMENTS

1. "Disclosed Exemplifications"

The Examiner alleges that the scope is excessive in view of the "disclosed exemplifications." Applicant respectfully disagrees. The specification discloses androgen receptor modulator compounds, pharmaceutical compositions containing such compounds as well as methods of using such compounds and pharmaceutical compositions for modulating processes mediated by steroid receptors. The application discloses methods of making such compounds and pharmaceutical compositions, as well as intermediates used in their synthesis. The specification describes seven generic synthesis schemes (for example, see page 33, 35, 37, 38, 39, 41 and 42). One of skill in the art can readily follow these schemes or known variations of such schemes with any of a vast number of commonly available starting materials to arrive at the claimed subject matter. The application names over 150 exemplary AR modulator compounds (for example, see page 29 through 32 and claims 56 and 57. The specification also provides over 50 working examples. The application teaches *in vitro* assays, such as the "cis-trans" or "co-transfection" assay, for characterizing exemplary AR modulator compounds. The specification provides *in vitro* binding data of exemplary compounds disclosed in the instant application that exhibit androgen receptor agonist activity, partial agonist activity, or antagonist activity. The compounds of the instant application are further characterized for their specificity for the androgen receptor by examining the *in vitro* binding activity with other members of the

steroid receptors. Hence the specification provides a variety of examples of compounds that fall within the scope of the claims evidencing that the claimed compounds function as claimed. The requirements of 35 U.S.C. §112, first paragraph, do not require a specific example of everything within the scope of the claims. *In re Anderson*, 176 USPQ 331, 333 (CCPA 1973) :

> ...we do not regard section 112, first paragraph, as requiring a specific example of everything within the scope of a broad claim . . . What the Patent Office is here apparently attempting is to limit all claims to the specific examples, not withstanding the disclosure of a broader invention. This it may not do.

In re Grimme, Keil and Schmitz, 124 USPQ 449, 502 (CCPA 1960) :

> It is manifestly impracticable for an applicant who discloses a generic invention to give an example of every species falling within it, or even to name every such species. It is sufficient if the disclosure teaches those skilled in the art what the invention is and how to practice it.

Hence there is no requirement for the applicant to exemplify or even provide an example of everything within the scope of the claims. The Patent Office cannot "limit all claims to the specific examples, notwithstanding the disclosure of a broader invention." A patentee's invention may be broader than the particular embodiment shown in the specification. A patentee not only is entitled to narrow claims particularly directed to the preferred embodiment, but also to broad claims that define the invention without a reference to specific instrumentalities. *Smith v. Snow*, 294 U.S. 1, 11, 24 USPQ 26, 30 (1935). Applicant is entitled to claims that are commensurate in scope not only with what applicant has specifically exemplified, but commensurate in scope with that which one of skill in the art could obtain by virtue of that which the applicant has disclosed.

2. "Defined by the Prior Art"

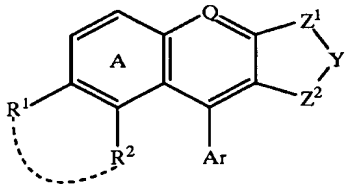
In the present Action, the Examiner alleges that "the state of the prior art is defined by the prior art presently cited by the Applicant and by Examiner and particularly by PTO-892 references F and G wherein anticipatory compounds and compositions have been disclosed." Applicant respectfully disagrees. The prior art is replete with references that show that the skilled artisan can use known organic synthesis schemes and reactions to produce various bicyclic, tricyclic and polycyclic organic compounds, including, for example, quinolines, quinolinones, coumarins, benzoxyzins, oxazolidines, azasteroids, progesterones, azachlormadinones, anthrasteroids, flutamides and phthalimides, and that the art of record teaches bioassays for evaluating whether compounds are functional ligands for receptor proteins and correlates the activity of ligands in such assays to *in vivo* activity (*e.g.*, see Evans *et al.* (US Pat. Nos. 4,981,784 and 5,071,773 and Science 240:889-95 (1988)).

Applicant disagrees that "the state of the prior art is defined by the prior art presently cited by applicant and by examiner." Applicant is not aware of such a definition and respectfully requests that the Examiner cite authority.

3. Alleged Anticipatory Prior Art

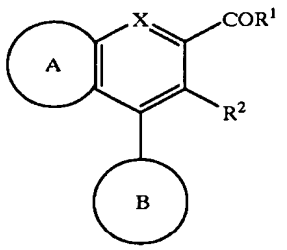
In the present Action, the Examiner alleges that certain references are anticipatory prior art (Action at page 4, which states that PTO-892 references F and G disclose anticipatory compounds and compositions). Reference F is US 6,030,967 (2/29/00) to Mauri *et al.* and Reference G is US 6,340,704 (1/22/02) to Mauri *et al.* Applicant respectfully submits that the Examiner has not rejected any of the claims under 35 U.S.C. § 102(b) as anticipated by either US Pat. Nos. 6,030,967 or 6,340,704. Applicant respectfully submits that neither US Pat. No. 6,030,967 nor US Pat. No. 6,340,704 discloses compounds as instantly claimed nor compositions thereof.

For example, US Pat. No. 6,030,967 discloses compounds having the formula:



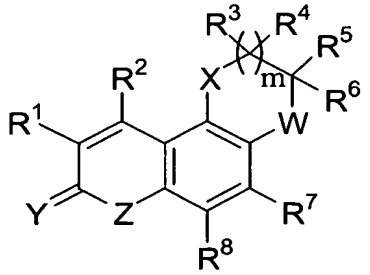
where Q is an optionally substituted carbon atom or $N(O)_p$ wherein p is 0 or 1; Y is an optionally substituted methylene group, $S(O)_q$ wherein q is an integer of 0 to 2, or an optionally substituted imino group; Z^1 is a C_{1-3} alkylene group which can have an oxo group or a thioxo group and can contain etherified oxygen or sulfur within the carbon chain; Z^2 is an optionally substituted C_{1-3} alkylene group; Ar is an optionally substituted carbocyclic group or an optionally substituted heterocyclic group; one of R^1 and R^2 is a hydrogen atom, a halogen atom, a hydroxyl group, an optionally substituted lower alkyl group, or an optionally substituted lower alkoxy group; the other is a halogen atom, a hydroxyl group, an optionally substituted lower alkyl group, or an optionally substituted lower alkoxy group; or R.sup.1 and R.sup.2 taken together with adjacent –c=c– form a ring; and ring *A* is a benzene ring which may be substituted in addition to R^1 and R^2 ; or a salt thereof.

US Pat. No. 6,340,704 discloses compounds having the formula:



where R^1 is an amino group that may be substituted, R^2 is a hydrogen atom or a lower alkyl group that may be substituted; X is a methane group that may be substituted or $N(O)_m$ (m is 0 or 1); ring *A* is a homo- or hetero-cycle that is substituted by a halogen atom, lower alkyl, lower alkoxy or lower alkylenedioxy group, and ring *B* is a homo- or hetero-cycle that may be substituted.

Neither US Pat. No. 6,030,967 nor US Pat. No. 6,340,704 discloses compounds or compositions as instantly claimed, such as those that have the structure set forth as formula I



I

and defined in the instant claims. Applicant also respectfully submits that the Office Action is internally inconsistent. At Paragraph C on Page 4 of the Action the Examiner alleges that PTO-892 References F and G disclose anticipatory compounds and compositions, while in Paragraph D on Page 4 of the Action, the Examiner states that these references disclose "many compounds very closely analogous to the instant claimed compounds." Further, on page 6 of the Action, the Examiner states that "claims 1-31, 37-42, 45, 46, 49-51, 58-72 and 75-77 would be allowable if rewritten or amended to overcome the rejection under 35 U.S.C. 112" (see last sentence on page 6). Thus, it appears that the Examiner is mistaken when he states on page 4 of the Action that US Pat. No. 6,030,967 and US Pat. No. 6,340,704 disclose anticipatory compounds and compositions. In order to clarify the record of the instant prosecution history, Applicant respectfully requests that the Examiner withdraw the statement that US Pat. No. 6,030,967 and US Pat. No. 6,340,704 disclose anticipatory compounds and compositions.

4. The instant claims include terms that allegedly are incompletely defined

The Examiner alleges that the compounds and pharmaceutical composition claims are enabled only in part because the instant claims include terms that allegedly are incompletely defined. The prior Office Action raised this issue under 35 U.S.C. § 112, second paragraph, and it has not been maintained in the instant Action. Thus, it appears that the rejection under 35 U.S.C. § 112, second paragraph has been withdrawn. Notwithstanding this, in order to be fully responsive, Applicant respectfully traverses the rejection.

a. aryl

The Examiner alleges that the term "aryl" is incompletely defined because "said terms typically i) lack any upper bounds as to size." Applicant respectfully disagrees.

It is respectfully submitted that the specification specifically defines the term "aryl." For example, page 9, line 26 through page 10, line 5, recites:

> The term "aryl," alone or in combination, refers to an optionally substituted aromatic ring system. The term aryl includes monocyclic aromatic rings, polyaromatic rings and polycyclic aromatic ring systems containing from six to about twenty carbon atoms. The term aryl also includes monocyclic aromatic rings, polyaromatic rings and polycyclic ring systems containing from 6 to about 12 carbon atoms, as well as those containing from 6 to about 10 carbon atoms. The polyaromatic and polycyclic aromatic rings systems may contain from two to four rings. Examples of aryl groups include, without limitation, phenyl, biphenyl, naphthyl and anthryl ring systems.

This definition includes upper bounds as to size. Applicant respectfully submits that claims must be read in view of the specification. See, *e.g.*, MPEP § 2106 ("An applicant is entitled to be his or her own lexicographer, and in many instances will provide an explicit definition for certain terms used in the claims. Where an explicit definition is provided by the applicant for a term, that definition will control interpretation of the term as it is used in the claim."); MPEP § 2173.05 ("When the specification states the meaning that a term in the claims is intended to have, the claim is examined using that meaning in order to achieve a complete exploration of the applicant's invention and its relation to the prior art."). The term "aryl" is expressly defined in the specification, and the definition recites specifics that the Examiner alleges to be missing ("bounds as to size"). Thus, the term "aryl" is not incompletely defined. Therefore, reciting the definition for "aryl" in the claims is not necessary.

b. arylalkyl

The Examiner alleges that the term "arylalkyl" is incompletely defined because "said terms typically i) lack any upper bounds as to size." Applicant respectfully disagrees. The term "arylalkyl" is defined in the specification. For example, see page 11, lines 9-11, which recites:

> The term "arylalkyl," alone or in combination, refers to an alkyl radical as defined above in which one hydrogen atom is replaced by an aryl radical as defined above, such as, for example, benzyl, 2-phenylethyl and the like.

The term "arylalkyl" is expressly defined in the specification, and references the terms "alkyl" and "aryl." As discussed above, the specification defines the recitation "aryl" (*e.g.*, see page 9, line 26 through page 10, line 5). In addition, the specification defines the recitation "alkyl" (*e.g.*, see page 8, lines 13-18):

> an optionally substituted straight-chain or branched-chain alkyl radical having from 1 to about 12 carbon atoms. The term also includes substituted straight-chain or branched-chain alkyl radicals having from 1 to about 6 carbon atoms as well as those having from 1 to about 4 carbon atoms. Examples of alkyl radicals include methyl, ethyl, *n*-propyl, isopropyl, *n*-butyl, isobutyl, *sec*-butyl, *tert*-butyl, *tert*-amyl, pentyl, hexyl, heptyl, octyl and the like.

The definitions provided in the specification for the terms "alkyl" and "aryl" recites the "upper bounds as to size" that the Examiner alleges to be missing. Thus, one of skill in the art would be apprised of the metes and bounds of the term "arylalkyl" when read in light of the specification. Therefore, reciting the definition for "arylalkyl" in the claims is not necessary.

c. heteroaryl

The Examiner alleges that the term "heteroaryl" is incompletely defined because:

> said terms typically
> i) lack any upper bounds as to size,
> and when heteroatoms are suggests said terms
> ii) fail to define which hetero atoms are to be selected from
> iii) the number of said heteroatoms, or
> iv) the location(s) or the ring system(s) containing said heteroatom(s) and
> v) because a proper definition of "optionally substituted" is not present in any independent claim.

Applicant respectfully submits that the specification defines the recitation "heteroaryl" (*e.g.*, see page 10, lines 6-19):

> optionally substituted aromatic ring systems containing from about five to about 20 skeletal ring atoms and having one or more heteroatoms such as, for example, oxygen, nitrogen and sulfur. The term heteroaryl also includes optionally substituted aromatic ring systems having from 5 to about 12 skeletal ring atoms, as well as those having from 5 to about 10 skeletal ring atoms. The term heteroaryl may include five- or six-membered heterocyclic rings, polycyclic heteroaromatic ring systems and polyheteroaromatic ring systems where the ring system has two, three or four rings. The terms heterocyclic, polycyclic heteroaromatic and polyheteroaromatic include ring systems containing optionally substituted heteroaromatic rings having more than one heteroatom as described above (*e.g.*, a six membered ring with two nitrogens), including polyheterocyclic ring systems of from two to four rings. The term heteroaryl includes ring systems such as, for example, furanyl, benzofuranyl, chromenyl, pyridyl, pyrrolyl, indolyl, quinolinyl, N-alkyl pyrrolyl, pyridyl-N-oxide, pyrimidoyl, pyrazinyl, imidazolyl, pyrazolyl, oxazolyl, benzothiophenyl, purinyl, indolizinyl, thienyl and the like.

The definition set forth in the specification for the term "heteroaryl" recites the "upper bounds as to size" that the Examiner alleges to be missing. The definition also states that the rings include one or more heteroatoms, such as oxygen, nitrogen and sulfur. Thus, Applicant respectfully submits that one of skill in the art, in light of what is known in the art and the teachings of the

specification, would understand what is meant by the recitation "heteroaryl" and would be able to determine the metes and bounds of the claims. Thus, reciting the definition for "heteroaryl" in the claims is not necessary.

d. "optionally substituted"

The term "optionally substituted" is defined in the specification at page 11, line 26 to page 12, line 9, which states:

> "Optionally substituted" groups may be substituted or unsubstituted. The substituents of an "optionally substituted" group may include, without limitation, one or more substituents independently selected from the following groups or designated subsets thereof: alkyl, alkenyl, alkynyl, heteroalkyl, haloalkyl, haloalkenyl, haloalkynyl, cycloalkyl, aryl, heteroaryl, arylalkyl, heteroarylalkyl, alkoxy, aryloxy, haloalkoxy, amino, alkylamino, dialkylamino, alkylthio, arylthio, heteroarylthio, oxo, carboxyesters, carboxamido, acyloxy, hydrogen, F, Cl, Br, I, CN, NO_2, NH_2, N_3, $NHCH_3$, $N(CH_3)_2$, SH, SCH_3, OH, OCH_3, OCF_3, CH_3, CF_3, $C(O)CH_3$, CO_2CH_3, CO_2H, $C(O)NH_2$, OR^9, SR^9 and $NR^{10}R^{11}$. An optionally substituted group may be unsubstituted (e.g., $-CH_2CH_3$), fully substituted (e.g., $-CF_2CF_3$), monosubstituted (e.g., $-CH_2CH_2F$) or substituted at a level anywhere in-between fully substituted and monosubstituted (e.g., $-CH_2CF_3$).

Thus, the recitation "optionally substituted" does not imply "that the unnamed substituents is/are open to all possible alternatives" as alleged by the Examiner. As discussed above, claims must be read in view of the specification. Where an explicit definition is provided by the applicant for a term, that definition will control interpretation of the term as it is used in the claim. The term "optionally substituted" is expressly defined in the specification. Thus, reciting that definition in the claims is not necessary.

Furthermore, the USPTO recognizes the use of this term in patent claims. A search of the USPTO database for the time period 1976 to present for patents with the recitation "optionally substituted" in the claims yielded 22,359 patents. While applicant realizes that the prosecution history of one patent is not relevant to another, the widespread use of the recitation "optionally substituted" in claims evidences that one of skill in the art understands the meaning of this term.

Notwithstanding the above, without acquiescing to the Examiner's allegation and solely to expedite issuance of the application, independent claims 1 and 58 are amended herein to recite the substituents encompassed by the recitation "optionally substituted" in the claims.

5. Spiro structures

The Examiner alleges that no heterocyclic or homocyclic spiro examples are included in any of the synthetic schemes or any of the specific compounds in the application.

Without a acquiescing to the Examiner's allegation and solely to expedite prosecution, claim 10 is cancelled herein without prejudice or disclaimer and claims 1, 9, 29-31, 49, 50, 58, 63, 71 and 72 are amended to delete recitations where two particular substituents together form a carbocyclic or heterocyclic ring. Thus, the rejection is moot. Applicant expressly reserves the right to pursue the cancelled subject matter in a continuing application.

6. The definitions of variables X and Z

The Examiner suggests that the definitions of variables "X" and "Z" should be separated. Without a acquiescing to the Examiner's allegation and solely to expedite prosecution and advance the application to issuance, claims 1 and 58 are amended herein to define the variables "X" and "Z" separately.

REJECTION OF CLAIMS 1-7, 9, 11-18, 20-25, 27-30, 49, 58, 60-62, 64-71, 73 AND 74 UNDER 35 U.S.C. §112, SECOND PARAGRAPH

Claims 1-7, 9, 11-18, 20-25, 27-30, 49, 58, 60-62, 64-71, 73 and 74 are rejected under 35 U.S.C. 112, second paragraph, as being indefinite for failing to particularly point out and distinctly claim the subject matter that applicant regards as the invention because the Examiner alleges that the recitation "optionally substituted" fails "to specify the substituents implied thereby." The Examiner also alleges that the definition for "optionally substituted" in the specification is "inadequate because the definition fails to meet the requirements of the statute for a variety of reasons noted in examiner's response following a previous rejection."

Applicant respectfully traverses the bases for the rejection in turn below.

RELEVANT LAW

Claims are not read in a vacuum but instead are considered in light of the specification and the general understanding of the skilled artisan. *Rosemount Inc. v. Beckman Instruments, Inc.*, 727 F.2d 1540, 1547, 221 USPQ 1, 7 (Fed. Cir. 1984). Claim language is satisfactory if it reasonably apprises those of skill in the art of the bounds of the claimed invention and is as precise as the subject matter permits. *Shatterproof Glass Corp. v. Libby-Owens Ford Col.*, 758 F.2d 613, 624, 225 USPQ 634, 641 (Fed. Cir.), *cert. dismissed*, 106 S.Ct. 340 (1985).

ANALYSIS

<u>"Optionally substituted"</u>

As discussed above, the term "optionally substituted" is defined in the specification (*e.g.*, see page 11, line 26 to page 12, line 9). Claims must be read in view of the specification. Where an explicit definition is provided by the applicant for a term, that definition will control

interpretation of the term as it is used in the claim (see *e.g.*, MPEP § 2106). The term "optionally substituted" is defined in the specification and thus that definition will control interpretation of the claim. Thus, reciting the definition in the claims is not necessary.

Notwithstanding this, without a acquiescing to the Examiner's allegation and solely to expedite issuance of the application, independent claims 1 and 58 are amended herein to recite the substituents encompassed by the recitation "optionally substituted" in the claims.

Alleged "Inadequate" Definition

The Examiner does not distinctly recite the alleged deficiencies in the definition for the recitation "optionally substituted" provided in the specification. Applicant respectfully requests that the Examiner restate the rejection with particularity to afford the applicant an opportunity to properly respond. In order to be fully responsive, Applicant provides the following traverse.

The Examiner objects to the definition of "optionally substituted" recited in the specification because it includes the terms "aryl," "arylalkyl" and "heteroaryl" because:

> said terms typically
> i) lack any upper bounds as to size,
> and when heteroatoms are suggests said terms
> ii) fail to define which hetero atoms are to be selected from
> iii) the number of said heteroatoms, or
> iv) the location(s) or the ring system(s) containing said heteroatom(s) and
> v) because a proper definition of "optionally substituted" is not present in any independent claim.

a. aryl

The specification specifically defines the term "aryl." For example, page 9, line 26 through page 10, line 5, recites:

> The term "aryl," alone or in combination, refers to an optionally substituted aromatic ring system. The term aryl includes monocyclic aromatic rings, polyaromatic rings and polycyclic aromatic ring systems containing from six to about twenty carbon atoms. The term aryl also includes monocyclic aromatic rings, polyaromatic rings and polycyclic ring systems containing from 6 to about 12 carbon atoms, as well as those containing from 6 to about 10 carbon atoms. The polyaromatic and polycyclic aromatic rings systems may contain from two to four rings. Examples of aryl groups include, without limitation, phenyl, biphenyl, naphthyl and anthryl ring systems.

This definition includes upper bounds as to size. Thus, the term "aryl" is not incompletely defined.

b. arylalkyl

The term "arylalkyl" is defined in the specification. For example, see page 11, lines 9-11, which recites:

> The term "arylalkyl," alone or in combination, refers to an alkyl radical as defined above in which one hydrogen atom is replaced by an aryl radical as defined above, such as, for example, benzyl, 2-phenylethyl and the like.

The terms "alkyl" and "aryl" are defined in the specification. As discussed above, the definition for the term "aryl" recites the "upper bounds as to size" that the Examiner alleges to be missing. Thus, the term "arylalkyl" is not incompletely defined.

c. heteroaryl

The specification defines the recitation "heteroaryl" (*e.g.*, see page 10, lines 6-19):

> optionally substituted aromatic ring systems containing from about five to about 20 skeletal ring atoms and having one or more heteroatoms such as, for example, oxygen, nitrogen and sulfur. The term heteroaryl also includes optionally substituted aromatic ring systems having from 5 to about 12 skeletal ring atoms, as well as those having from 5 to about 10 skeletal ring atoms. The term heteroaryl may include five- or six-membered heterocyclic rings, polycyclic heteroaromatic ring systems and polyheteroaromatic ring systems where the ring system has two, three or four rings. The terms heterocyclic, polycyclic heteroaromatic and polyheteroaromatic include ring systems containing optionally substituted heteroaromatic rings having more than one heteroatom as described above (*e.g.*, a six membered ring with two nitrogens), including polyheterocyclic ring systems of from two to four rings. The term heteroaryl includes ring systems such as, for example, furanyl, benzofuranyl, chromenyl, pyridyl, pyrrolyl, indolyl, quinolinyl, N-alkyl pyrrolyl, pyridyl-N-oxide, pyrimidoyl, pyrazinyl, imidazolyl, pyrazolyl, oxazolyl, benzothiophenyl, purinyl, indolizinyl, thienyl and the like.

The definition set forth in the specification for the term "heteroaryl" recites the "upper bounds as to size" that the Examiner alleges to be missing. The definition also states that the rings include one or more heteroatoms, such as oxygen, nitrogen and sulfur. Thus, Applicant respectfully submits that one of skill in the art, in light of what is known in the art and the teachings of the specification, would understand what is meant by the recitation "heteroaryl" and would be able to determine the metes and bounds of the claims. Thus, the term "heteroaryl" is not incompletely defined.

REBUTTAL TO THE EXAMINER'S ARGUMENTS

The Definition of Variable R^{18}

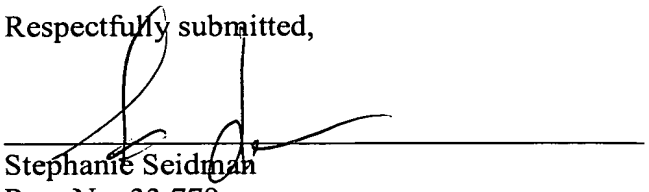
In the rejection under 35 U.S.C. 112, second paragraph, the Examiner alleges the recitation "may be optionally substituted" has been replaced with the recitation "optionally substituted" in the claims except in the case of variable R^{18} in claims 1 and 58. Without

addressing the propriety of the rejection, Applicant respectfully submits that neither claim 1 nor claim 58 recites a variable R^{18}. Thus, because claims 1 and 58 do not recite a variable R^{18}, this rejection is moot.

* * *

In view of the above, reconsideration and allowance of the application are respectfully requested.

Respectfully submitted,



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Attorney's Docket No.: 18202-018001 / 1082

RESPONSE UNDER 37 CFR §1.116 -- EXPEDITED PROCEDURE -- EXAMINING GROUP 1600

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant : Lin Zhi *et al.*
Serial No. : 10/080,503
Filed : February 22, 2002
Title : **TRICYCLIC QUINOLINONE AND TRICYCLIC QUINOLINE ANDROGEN RECEPTOR MODULATOR COMPOUNDS AND METHOD**

Art Unit : 1623
Examiner : Lawrence E. Crane, Ph.D.

MAIL STOP AF
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

SUPPORTING DOCUMENTS

1. Rosen *et al.*, J. Med. Chem. 38(25): 4855-4874 (1995).
2. TESTODERM (Brand name drug), NDA 019762, Approval History, US Food and Drug Administration; Approval – October 12, 1993.
3. ANDRODERM (Brand name drug), NDA 020489, Approval History, US Food and Drug Administration; Approval – September 29, 1995.
4. ANADROL-50 (Brand name drug), NDA 016848, Approval History, US Food and Drug Administration; Approval – January 18, 1972.
5. TESTRED (Brand name drug), ANDA 083976, Approval History, US Food and Drug Administration; Approval – December 3, 1973.
6. EULEXIN (Brand name drug), NDA 018554, Approval History, US Food and Drug Administration; Approval – January 27, 1989.
7. CASODEX (Brand name drug), NDA 020498, Approval History, US Food and Drug Administration; Approval – October 4, 1995.
8. Gruber *et al.*, Arch Dermatol 134: 459-463 (1998).
9. Venturoli *et al*, J Clin Endocrinology & Metabolism 84(4): 1304-1310 (1999).
10. Berger *et al.*, J. Steroid Biochem. Molec. Biol. 41: 773-738 (1992).

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Perspective

Intracellular Receptors and Signal Transducers and Activators of Transcription Superfamilies: Novel Targets for Small-Molecule Drug Discovery

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Introduction

The proliferation and differentiation of mammalian cells is modulated by a number of specific signal molecules that regulate gene expression. Among these signals are (i) the steroid hormones (*e.g.*, glucocorticoids, mineralocorticoids, estrogens, progestins, and androgens), chemical messengers produced by the body in response to a variety of stimuli; (ii) small-molecule hormones including thyroid hormone, calcitriol (a vitamin D_3 metabolite), and the retinoids; and (iii) the cytokine superfamily of protein molecules that affect cells of the immune and other systems.

The mechanism of action of steroid hormones has been studied extensively over the last 25 years. The steroid hormones, which have similar fused ring skeletons, vary structurally with the placement of double bonds and the nature of their side chains. Dramatic differences in biological activity of these molecules are due to interactions with specific receptors capable of distinguishing minor differences in chemical structure. In contrast, nonsteroidal small-molecule hormones vary significantly in chemical structure; however, they utilize similar receptors to produce their biological effects. The receptors for the nonsteroidal small-molecule hormones include the retinoic acid (RA) receptor subtypes (RARα, RARβ, RARγ, RXRα, RXRβ, and RXRγ), the vitamin D receptor, and the thyroid hormone receptor subtypes (T_3Rα and T_3Rβ and splice variants).

The definition of biochemical events that mediate signal transduction in response to steroid hormones and small-molecule hormones has advanced rapidly, beginning in 1985 with the first cloning of an intracellular receptor (IR).[1] Molecular biological techniques enabled subsequent cloning and characterization of receptors for each of the steroid and small-molecule hormones. This dramatically enhanced understanding of hormone action and led to a number of unifying insights concerning the receptors and their ligands. The IRs are closely related members of a protein superfamily[2] that have apparently diverged from a common ancestral gene.[3] The presence of a specific IR within a cell enables that cell to respond to the hormone cognate to that IR. The IRs share a common mechanism of action, since they in general remain latent inside target cells until exposed to their specific ligands, which activate them as transcription factors producing specific changes in gene expression.

Cytokines are a large and diverse family of circulating polypeptides produced by many different cell types. They include various types of interferons (*e.g.*, IFN-α, -β, -γ), the interleukins (*e.g.*, IL-6), the colony-stimulating factors (*e.g.*, granulocyte colony-stimulating factor, G-CSF), and growth factors (*e.g.*, epidermal growth factor, EGF). Individual cytokines act upon a variety of cell types. As polypeptides, cytokines cannot freely enter cells; they act by binding to specific cell surface receptors. The understanding of the biochemical events by which some of the cytokines achieve their distinctive biological effects has increased significantly since 1992. There is a surprising degree of underlying similarity in the pathways of cytokine signal transduction, explained by the discovery of a family of latent cytosolic proteins, termed signal transducers and activators of transcription (STATs),[4] that mediate signal transduction for the majority of the cytokines. This newly defined STAT protein superfamily acts to mediate specific changes in gene expression and consequently cell function following exposure to most cytokines.

Both the IRs and the STATs act as DNA-sequence-

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specific transcription regulators, selecting the genes expressed and modulating the level of their expression within a cell following exposure to a specific stimulus. The DNA sequence-specific factors that act to modulate gene transcription are collectively termed transcription factors and include the IRs and STATs, which exert their effects by binding to chromosomal DNA in a sequence-specific manner or by interacting with components of the transcription apparatus, the complex of RNA polymerases and accessory proteins that carry out the production of messenger RNA (mRNA). Transcription factors control the expression of specific sets of genes. Since the pattern of gene expression determines cell function, the control of gene expression is a central process in biology.

The concepts and terminology used to describe the transcription of eukaryotic genes will be briefly reviewed here, preceding a detailed discussion of the mechanics of the IR and STAT signal transduction pathways and their relevance to drug discovery. Transcription, the rate-limiting step in gene expression, is used by the cell as a primary point of regulation for subsequent events controlled by hormones and cytokines. The regulatory pathways involved in transcription are controlled by protein–protein and protein–DNA interactions.

Transcriptional activation of eukaryotic genes during development or in response to extracellular signals is a complex process involving the concerted action of many proteins. RNA polymerase II is the enzyme responsible for the production of mRNA from genes in eukaryotes, as outlined in Figure 1.[5] It acts in combination with a number of transcription factors. These factors can be divided into three classes on the basis of their functions. First are the basal transcription factors, which are required for an unregulated, basal level of transcription by RNA polymerase II. Second are the DNA-sequence-specific transcription factors, which are required for regulated transcription of a subset of these genes. Lastly, the coactivators represent a newly discovered class of regulatory proteins that act in concert with sequence-specific and basal transcription factors to further modulate levels of transcription.

The regulatory region in the immediate vicinity of the transcription start site is termed the promoter and contains a number of core response elements. Response elements are specific nucleotide sequences that are recognized by and act as binding sites for transcription factors. The core response elements are usually located within several hundred base pairs of the transcription start site. The most common core response element among genes transcribed by RNA polymerase II is the 5′-TATAAA-3′ sequence (TATA box), recognized by a specific basal transcription factor. Other response elements commonly found in the promoter region and to which specific transcription factors bind include the 5′-GGGCG-3′ and 5′-CCAAT-3′ sequences. In addition, mammalian genes may contain particular combinations of positive or negative regulatory response elements that are uniquely arranged as to number, type, and spatial organization. These response elements are the binding sites for sequence-specific transcription factors that activate or repress gene expression for the gene downstream from that promoter. A response element that regulates the activity of the promoter from a distance and in an orientation-independent fashion is termed an enhancer or a silencer depending on whether it induces or represses gene expression. Both the IRs and STATs interact with response elements to control transcription.

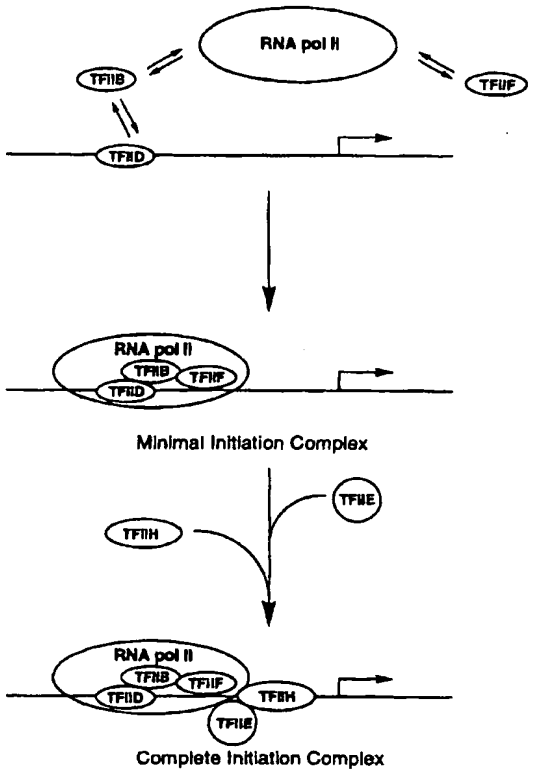


Figure 1. The cascade of events leading to transcriptional initiation. The first step in the initiation process requires binding of TFIID to the TATA box of the promoter. After TFIIB binds to TFIID on the DNA, RNA polymerase II and TFIIF are recruited to the complex of proteins at the transcription initiation start site. Transcriptional initiation also requires the presence of TFIIE and TFIIH to yield a complete initiation complex.

Initiation of transcription requires that the basal transcription factors, by interaction with core response elements, form an initiation complex, the active assemblage of RNA polymerase II and accessory proteins required to start RNA synthesis. A schematic representation of initiation complex formation is shown in Figure 1. The first step in the assembly of the initiation complex is the binding of the transcription factor D for RNA polymerase II (TFIID)[6] to the TATA box. TFIID is a multiprotein complex that consists of the TATA binding protein (TBP) and TBP associated factors. TFIID acts as a binding site for TFIIB. Once bound, TFIIB is able to recruit RNA polymerase II and TFIIF to the transcription start site. The complex formed by the association of these factors is stable; however, subsequent association of transcription factors TFIIE and TFIIH is required to complete formation of the transcription initiation complex to begin mRNA production.[5,7]

Sequence-specific transcription factors modulate the formation of the initiation complex and thus control the frequency of transcription of a specific subset of genes

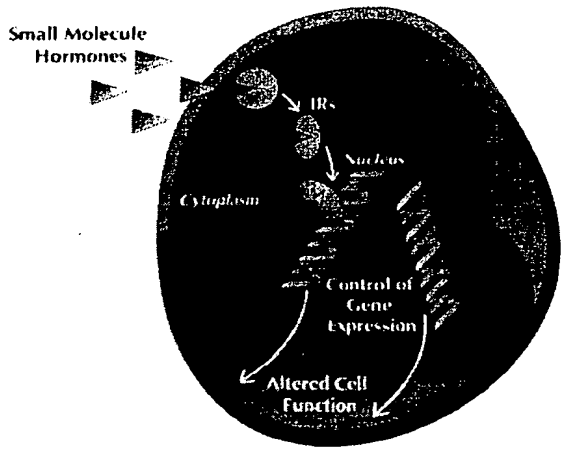


Figure 2. Control of gene expression through the intracellular receptor signaling pathway. In the IR pathway, small-molecule ligands diffuse into the cell and bind to the appropriate receptor. This leads to a conformational change of the receptor that causes dissociation with proteins such as hsp90 that are associated with the inactive form of the receptor and allows receptor binding to a specific response element in the promoter of a gene controlled by that hormone. Binding of an activated IR protein to its response element modulates the transcription of the downstream gene and, thus, translation of the gene product.

by RNA polymerase II. These transcription factors may act (i) by binding to sequence-specific response elements (enhancers or silencers) within the DNA or (ii) *via* direct protein−protein interaction with basal transcription factors within the initiation complex.

Recently, coactivators have been identified as a third class of transcription factors. It has been proposed that these function as physical links between the sequence-specific transcription factors and one or more components of the initiation complex. The TFIID complex, for example, contains several proteins that are tightly associated with the TBP and display some coactivator function. These proteins may be unnecessary for basal level transcription but essential for stimulation of transcription by sequence−specific transcription factors.

The remainder of this review will focus on two families of sequence-specific transcription factors: (i) the IRs, the transcription factors through which steroid hormones and small-molecule hormones control gene expression, and (ii) the STATs, the transcription factors through which many cytokines and growth factors control gene expression.

Information regarding hormonal and cytokine signal transduction that has delineated the underlying principles, properties, and biological roles of IRs and STATs will be discussed. Important implications for new drug discovery approaches, targets, and tools with the potential to yield breakthrough small-molecule drugs mimicking or blocking hormone and cytokine actions will be highlighted.

Intracellular Receptor Function

The sequence of events involved in IR signal transduction is shown in Figure 2. Briefly, the non-peptide hormones, such as estrogen or RA, are sufficiently lipophilic to diffuse freely through the cell membrane without the need for specialized transport systems. A number of these hormones interact with plasma and intracellular binding proteins that show varying degrees of specificity; however, the actual mediators of hormonal signal transduction are the IRs. The intracellular receptors, proteins with molecular masses that range from approximately 55 to 90 kDa, have a characteristic domain structure. They are located within the cell, but are not cell membrane associated. Once a hormone enters the cell, it binds to its IR with high affinity, resulting in a conformation change in the receptor, activating the IR as a transcription factor.

In the absence of bound ligand, inactive steroid hormone receptors are sequestered in cells in a complex with the heat shock proteins hsp-90, hsp-70, and p59.[8] Additional proteins, such as YDJ1, also appear to influence the activation of steroid hormone receptors.[9] The cellular localization of the unliganded complex (cytoplasmic or nuclear) remains controversial.[10] The conformational change that occurs in the receptor as a consequence of hormone binding results in the dissociation of the IR from the heat shock proteins and release of the monomeric receptor molecule and its ligand from the complex. In contrast to the steroid hormone receptors, the inactive small-molecule hormone receptors do not appear to interact with heat shock proteins and, in the absence of hormone, are located in the nucleus.[11] The binding of hormone also results in conformational changes in these receptors and in their subsequent activation. The exact nature of these conformational changes is not known. However, for some of the IRs it can be shown to involve alteration in the accessibility of the IR's C-terminal region, detectable either with immunological reagents[12] or by determination of ligand modulation of proteolytic susceptibility *in vitro*.[13]

There are approximately 100000 genes expressed in all cells, with 10000−20000 expressed in a single cell. Of these, only a few hundred genes in any cell are regulated by IRs. Ligand-activated IRs exert their effects by binding directly to specific chromosomal enhancer sequences termed hormone response elements (HREs) that are located within the regulatory regions of target genes.[14,15] Once bound to HREs, the activated receptor increases the transcriptional activity of the adjacent promoter, resulting in optimal expression of the target gene. Each HRE is made up of two approximately hexanucleotide half-sites separated by a variable number of nucleotides. The sequence of the half-sites and the number, but not the sequence, of the spacing nucleotides are key determinants of the specificity of IR interaction. HREs differ in their nucleotide sequences as well as the orientation and spacing of their half sites. Comparison of the sequences of the HREs from different hormone responsive genes indicates that a similar motif is used by each of the receptor subfamilies (Table 1).

Recent evidence shows that the IRs can associate to form homodimers, heterodimers, and possibly other oligomeric receptor species.[16,17] These dimers may bind to inverted repeats, direct repeats, or everted repeats. It is generally believed, in the case of the glucocorticoid receptor (GR),[1] estrogen receptor (ER),[18] progesterone receptor (PR),[19,20] androgen receptor (AR),[21−23] and mineralocorticoid receptor (MR)[24] that the active receptor species are homodimers. For many of the other IRs, including thyroid hormone receptor (TR),[25] RA receptors

Table 1. Hormone Response Elements

Receptor		Example of HRE	Consensus Sequence
GR, MR,	MMTV	GTTACA AAC TGTTCT	GGTACANNN TGTTCT
AR & PR	TO	TGCACA GCG AGTTCT	
	TAT	TGTACA GGA TGTTCT	
ER	cVit	GGTCA GCG TGACC	GGTCANNN TGACC
	rPrl	TGTCA CTA TGTCC	
RXR	rCRBPII	AGGTCA C AGGTCA	AGGTCA N AGGTCA
VDR	hOST	GGGTGA ACG GGGGCA	AGGTCANNN AGGTCA
TR	hMHC	AGGTGA CAGG AGGACA	AGGTCANNNN AGGTCA
RAR	hRARβ	GGTTCA CCGAA AGTTCA	AGGTCANNNNN AGGTCA

MMTV, mouse mammary tumor virus; TO, tyrosine oxidase; TAT, tyrosine aminotransferase; cVit, chicken vitellogenin; rPrl, rat prolactin; rCRBPII, rat cellular retinol-binding protein type II; hOST, human osteocalcin; hMHC, human cardiac myosin heavy chain; hRARβ, human RARβ. "→ ←" inverted repeat of half-site. "→ →" direct repeat of half-site.

(RARs),[26,27] vitamin D receptor (VDR),[28] and a number of the orphan receptors, the functional transcription factor is a heterodimer formed with a member of the retinoid X receptor (RXR)[29,30] subfamily.

The idea that the receptors can only bind to HREs as dimers is being reexamined since the estrogen receptor appears able to bind as a monomer to a single half-site of either the estrogen response element or the thyroid hormone response element.[31,32] Further, two recently described proteins with significant primary sequence homology to members of the IR superfamily, nerve growth factor I-B (NGFI-B)[33,34] and steroidogenic factor-1 (SF-1),[35] also bind to half-sites as monomers. In each case, the HRE is an extended estrogen response element with extra 5′ nucleotides. Although not clearly understood, the selection of the DNA-binding mode appears to be determined by response element type, promoter context, and relative levels of the pertinent IRs.

Intracellular Receptor Structure

The cloning and sequencing of cDNAs for the IRs and comparison of their deduced amino acid sequences show that the superfamily members are modular in structure.[2,36] Sequence data and functional analysis show the IRs to consist of six discrete subdomains, A–F (Figure 3). Three of these domains have been described in detail: (i) the DNA-binding domain, which is highly conserved and provides specific binding to the HRE, (ii) the ligand-binding domain located C-terminal to the DNA-binding domain, which provides a hydrophobic pocket for the binding of the ligand but also contains a number of other functionally important regions, and (iii) the C- and N-terminal transactivation domains, which are more variable in sequence.

I. DNA-Binding Domain. The DNA-binding domain, which is usually centrally located in the primary sequence of an IR, is composed of 68 amino acid residues. Of those 68 amino acids, 20 are invariant and determine the generalized DNA-binding structure.[37] Confirmation that this region is the DNA-binding domain was obtained using XFACS,[38] 2D NMR,[39,40] and X-ray crystallography.[41] The DNA-binding domain is highly basic and contains nine cysteine amino acid residues. The presence of the cysteine residues enables the coordination of two zinc ions and the formation of two "zinc fingers". Selection of the specific HRE to which the receptor binds is determined by the three amino acids at the base of the first finger.[15] Mutational analysis of the estrogen receptor showed that conversion of these three amino acid residues to the corresponding residues from the glucocorticoid receptor results in a mutant estrogen receptor that binds the glucocorticoid response element.[42] The finger segment encompassing these residues, responsible for sequence specificity or selectivity of DNA binding, is termed the Proximal or P box.[43,44] The sequence spanning cysteine residues 5 and 6, in the second zinc finger, is responsible for discriminating between response elements with similar sequences but different half-site spacing. This sequence of five amino acid residues is called the Distal or D box.[45] For example, the D box in the RXR recognizes a one nucleotide spacing between the response element half-sites (5′-AGGTCA n AGGTCA-3′), while the RAR D box recognizes half-sites with five spacing nucleotides (5′-AGGTCA nnnnn AGGTCA-3′).

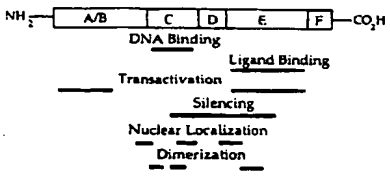


Figure 3. The domain structure of the intracellular receptors. The DNA-binding domain (DBD) corresponds to the C region and the ligand-binding domain (LBD) corresponds to the E region. These critical domains are restricted to defined segments of the protein. However, the segments of the receptor responsible for transactivation, nuclear translocation, and dimerization are not restricted to single defined domains, but are found in multiple regions of the receptor. The region marked D is a short region known as the hinge region that connects the DBD and the LBD and has functional activity as shown in the figure. The DBD contains nine cysteine residues enabling the coordination of two zinc ions and the formation of two zinc fingers. The first zinc finger contains the P box responsible for the sequence specificity of the IR; the second zinc finger of the DBD contains the D box, which discriminates between similar response element sequences with different spacing between the half-sites of the response element.

II. Ligand Binding Domain. The C-terminal or E region of the IRs is approximately 25 kDa and contains the ligand-binding domain (LBD), which determines the ligand-binding specificity of each receptor.[2,36] Proteolytic mapping of the receptor indicated that a portion of the D region is necessary for binding of ligand with maximal affinity.[12] The hormonal ligands generally bind to their cognate IRs with affinity constants (K_d values) on the order of 1 nM. Upon binding hormone, the receptor is thought to undergo a major conformational change that results in its activation.[12] The crystal structure of the RXRα ligand binding domain has been reported.[46] However, since the crystal structure was determined in the absence of a ligand, the conformational changes induced by hormone binding have not yet been clearly defined. The ligand-binding domain additionally contains regions allowing (i) the dimerization of the receptor monomers,[45,47,48] (ii) the interaction of selected IRs with heat shock proteins,[8] (iii) nuclear translocation signals,[49] and (iv) one of

several transcriptional transactivation domains of the receptor. The dimerization[50] and nuclear translocation[51] signals that have been mapped to the ligand binding domain are dependent upon the binding of hormone for their action. Nuclear translocation and dimerization signals are also present in the DNA-binding domain, but their action is hormone independent. Analysis of the region of the ligand binding domain that contains the dimerization signal has revealed a heptad repeat of hydrophobic residues that are highly conserved within the IR superfamily. This observation suggests that the nuclear hormone receptors dimerize via a leucine-zipper type mechanism.[52]

III. Transactivation Domains. Transactivation domains are located in both the N-terminal and C-terminal regions of the receptors. Transactivation achieved with the DNA-binding domain alone represents a small portion of the total activity of the receptor. Deletion of the E regions of GR and ER, however, abolishes both hormone-binding and transactivation functions of the receptors. Since nuclear localization is a prerequisite for transactivation, it is difficult to assess the influence of the E region independent of its nuclear translocation function. In addition, at least in the case of the estrogen receptor, transactivation has been shown to be dependent upon the binding of hormone.[53] The situation is further complicated by the possibility that dimerization of the receptor may be necessary for efficient transactivation. However, careful mutational analysis, together with experiments involving chimeric genes, has enabled identification of specific regions that contain the transactivation functions for a number of the IRs. The transactivation domains, located N-terminal and C-terminal to the DNA-binding domain, have been termed transactivation unit 1 and 2, respectively (Tau 1 and Tau 2), in GR,[54] transactivation function 1 and 2 (TAF-1 and TAF-2) in ER,[55] and activation function 1 and 2 (AF-1 and AF-2) in PR.[56] The Tau 1, TAF-1, and AF-1 activation domains are located within the region marked A/B in Figure 3; the second activation domain of these receptors can generally be found in the LBD. The retinoid receptors, RARs and RXRs, have also been reported to have two domains responsible for transactivation, one each in the N-terminal and C-terminal regions.[57]

Application of Intracellular Receptor Technology

Elucidation of the mechanism of IR-mediated transcriptional activation has enabled the development of high-throughput assays to detect novel small molecules that act as agonists, antagonists, or partial agonists of the IRs.[58] These assays can reveal the consequences of the interaction of any compound with any IR, using the cloned human IR cDNAs. These assays, termed cotransfection assays, are capable of detecting the functional effects on gene expression of small molecules that interact with specific IRs in mammalian cells. As shown in Figure 4, a gene for an IR that is a potential drug target is introduced by transfection (a procedure to induce cells to take up foreign DNA) into a mammalian cell lacking endogenous IRs of the type being studied. The introduced receptor cDNA directs the recipient cells to synthesize the receptor protein. A second gene under the transcriptional control of the appropriate HRE is also introduced by transfection (*i.e.*, cotransfected) into the same cells together with the IR gene. The protein product of this second gene functions as a reporter for the transcription-modulating activity of the receptor protein at its HRE. Thus, the reporter acts as a surrogate for the products normally expressed by genes under the control of the target receptor and its natural hormone. The reporter gene is chosen to encode a protein product that can be readily detected and quantified. Enzymes are useful reporters because they can often be assayed easily and, under the appropriate assay conditions, the rate of the reaction they catalyze directly reflects the amount of enzyme present. Firefly luciferase is an example of a frequently used reporter. The promoter that controls expression of the luciferase cDNA in the reporter plasmid is constructed to contain the appropriate HRE so that expression is under hormonal control at the level of transcription.

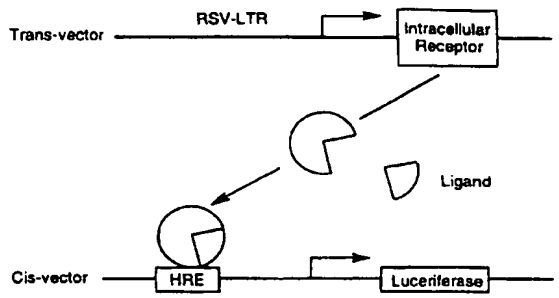


Figure 4. The cotransfection assay. Two plasmids are cotransfected into the appropriate cell background. One plasmid constitutively expresses an intracellular receptor. In the presence of ligand, the receptor acts in *trans* to bind to its HRE (the *cis* element) and activate transcription of the downstream reporter gene. The reporter is generally an easily detected enzyme such as chloramphenicol acetyl transferase (CAT) or luciferase.

The cotransfection assay can be used to detect agonists for the target IR. A day after exposure to an appropriate agonist ligand in the medium bathing the transfected cells, an increase in reporter activity can be measured in cell extracts, reflecting ligand-dependent, IR-mediated increases in reporter gene transcription.

The cotransfection assay can also be used to detected the activity of small molecules that antagonize the activity of an agonist ligand for any IR. To detect antagonists the assay is carried out in the presence of a constant concentration of a known agonist sufficient to induce a constant reporter signal. Cells incubated in the presence of increasing concentrations of an antagonist will display progressive decreases in reporter signal. The cotransfection assay is therefore useful to detect both agonists and antagonists of specific IRs.

Current Therapeutics

Hormone-based therapies have been part of clinical medicine for over a century. Initially, extracts of endocrine glands were used as replacement therapies to supplement patients with glandular deficiencies.

Subsequently, the hormones themselves, purified from such extracts, were administered for similar uses. As the structures of the hormones were determined, chemically-synthesized versions replaced many of the naturally-derived hormone drugs. Subsequently, chemical analogues of these were synthesized and tested in animals to find compounds with improved therapeutic profiles relative to those of the hormones. Hormone agonists in clinical use include estrogens, anti-inflammatory glucocorticoids such as cortisone and dexamethasone, thyroxine, vitamin D_3 (a precursor to calcitriol), various progestins, and estrogens used in oral contraception, and vitamin A metabolites.

The sex steroids drive the growth and function of the tissues of the reproductive tract and breasts. Malignancies arising in these organs, such as breast or prostate cancer, derive from normal tissue and often are dependent on sex steroids to maintain growth. Initially, these cancers were treated by surgical ablation of the endocrine glands involved in the secretion of the sex steroids. The surgical removal of the glands that produce an "unwanted" hormone is drastic and far from satisfactory. It was therefore a great advance when hormone antagonist drugs first became available. These hormone antagonists were developed, as were the early hormone agonists, by a laborious process of chemical synthesis of drug candidates and testing in animals. Steroid hormone antagonists with clinical utility include the anti-estrogen tamoxifen, the anti-progestin mifepristone (RU486), and the anti-androgens flutamide and cyproterone acetate. Other clinically useful hormone antagonists include the anti-mineralocorticoid spironolactone.

Identification of Targets for the Discovery of Novel Drugs

The number of diseases that are associated with inappropriate production of or response to hormonal stimuli highlights the medical and biological importance of these effectors. The recent advances in our understanding of the molecular basis for the action of IRs offer the opportunity to improve many of the existing IR-modulating drugs. Despite the clinical utility of currently available hormone agonists and antagonists, many of the compounds are limited by their side-effect profiles. Delineation of the mechanism of a specific biological response enables the identification of small molecules that retain the efficacy of the IR-modulating drug, but have significantly improved side-effect profiles. Development of compounds more selective for the target IR and, thus, in the function(s) that they elicit are discussed in section I, below.

The discovery of receptor isoforms for a variety of receptor systems has already led to the development of more specific and improved drugs. These include the cardioselective β-adrenergic blockers and the receptor subtype selective antihistamines. For certain non-protein hormones, identification of intracellular receptor subtypes, many of which are expressed in a tissue specific manner, not only implies a specific physiological role for each subtype but may offer better defined pharmacological effects and thus potential for development of highly selective drugs. The utility of this approach for drug discovery is detailed in section II, below.

Another recent finding, offering additional potential for the discovery of new drugs, is the identification of several dozen "orphan" members of the IR superfamily. In most cases, ligands that activate these so-called orphan receptors have yet to be identified. These IRs may represent as yet uncharacterized signal transduction pathways for novel endocrine and paracrine systems or may include subtypes of IRs for known ligands. In either case the orphan IRs, discussed in section III, below, are of great interest for discovery of more selective and efficacious small molecule drugs.

I. Tissue-Selective Intracellular Receptor Activators. Progesterone is produced in the ovaries, testes, adrenal cortex, and placenta. Along with estrogens, progesterone is critical in preparing the female reproductive tract for reception of sperm and implantation of a fertilized ovum. Progestins and estrogens cause growth and development of the reproductive tract and breasts. Progesterone is responsible for the body temperature rise upon ovulation and is also critical for the maintenance of pregnancy.

Therapeutic uses for progestins may include contraception (in combination with estrogens) and control of dysfunctional uterine bleeding, dysmenorrhea (in combination with an estrogen), endometriosis, and threatened spontaneous abortion. The traditional steroidal agonists of the progesterone receptor (*e.g.*, norgestrel and norethindrone) were synthesized over 25 years ago and exhibit anti-estrogenic activity along with varying degrees of cross reactivity with AR and GR.[59] While some cross reactivity with other receptors may occasionally be desirable,[60] opportunities now exist to prepare selective, easily synthesized progesterone receptor agonists and antagonists. Two functionally different approaches by which this may be achieved will now be discussed.

The recent discovery that the human progesterone receptor (hPR) exists in two forms, hPR-A (94 kDa) and hPR-B (120 kDa), opens one avenue for drug discovery. These receptors differ by 164 amino acids, which are present in the N-terminal region of hPR-B but absent from hPR-A. The two receptors have identical DNA and ligand binding domains and may[61] or may not[62] be present in equimolar concentrations in tissues. Hetero- and homodimers of hPR-A and hPR-B form upon ligand activation. Recent data suggest that the cellular pathways used by hPR-A and hPR-B are distinct.[63] The hPR-A has been demonstrated to inhibit the transcriptional activity of the glucocorticoid,[64] estrogen,[64] androgen, and mineralocorticoid[66] receptors in a cell- and promoter-specific manner. These properties may facilitate the identification of more selective compounds or compounds with cross reactivity by allowing *in vitro* analysis of progestin action. For example, hPR-A inhibition of ER may be a mechanism through which the anti-estrogenic properties of progestins are produced. These observations, if they apply *in vivo*, set the stage for discovering a new generation of hPR modulators.

Anti-progestins are currently used acutely as abortifacients. Other possible therapeutic uses may include cervical ripening and treatments for endometriosis, uterine fibroids, meningioma, and breast cancer. The anti-progestins are predominantly 11β-aryl-19-nor steroids[67], (*e.g.*, mifepristone, onapristone and Org 31806, Figure 5).[68-70] Cross reactivity with the androgen, glucocorticoid, and estrogen receptors is a feature of

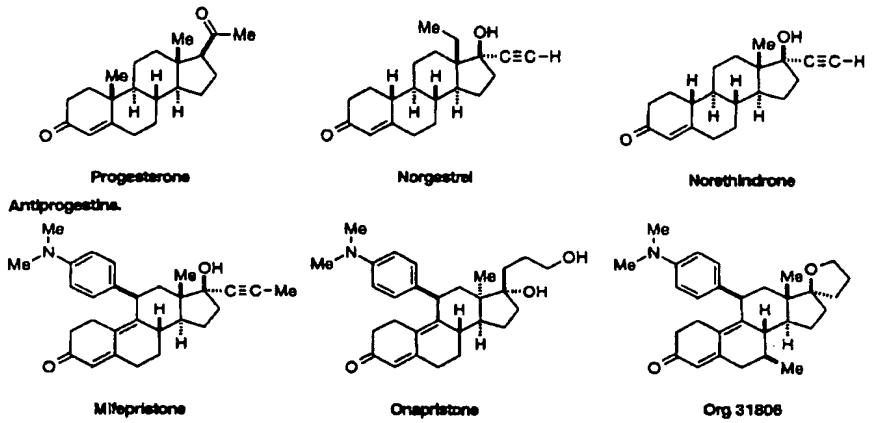


Figure 5. Progesterone–receptor agonists and antagonists.

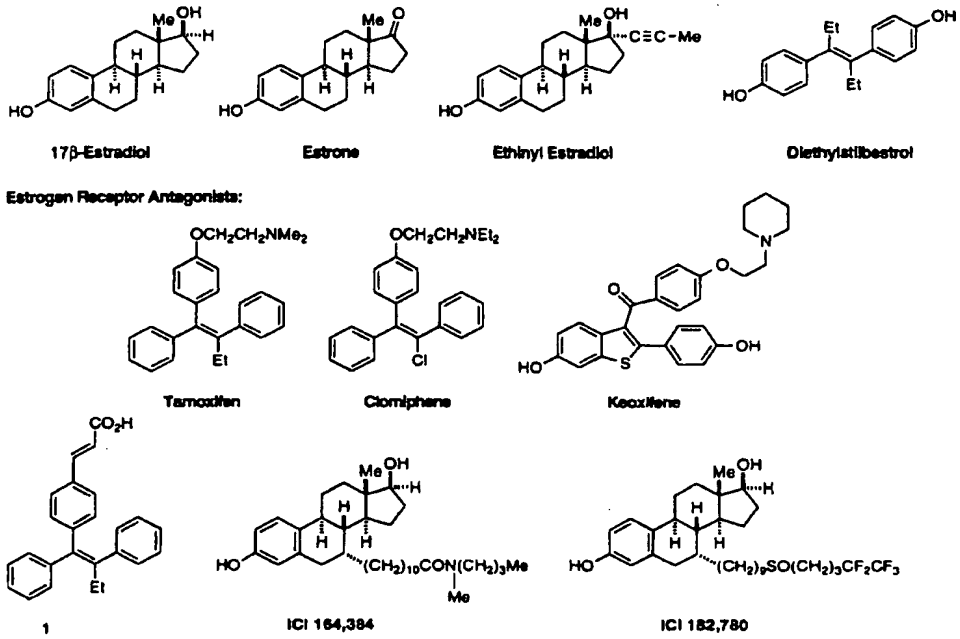


Figure 6. Estrogen receptor modulators.

current anti-progestins, which potentially limits their tolerability for chronic administration. Mifepristone[68] is a potent anti-progestin, anti-glucocorticold, and anti-androgen that also exhibits anti-estrogenic behavior. These cross reactivities, while of little consequence for acute uses, may be detrimental in chronic therapy.

Substantial progress has been made to prepare more selective 11β-aryl steroidal anti-progestins; however, these compounds are difficult to synthesize.[67] Furthermore, some evidence suggests that there are two classes of anti-progestins among the 11β-aryl steroids[71] that can be differentiated by the affinity of the ligand-bound dimerized receptor complex for DNA. Mifepristone and its analogues induce a dimerized receptor conformation with high affinity for DNA, while onapristone induces a conformation with low affinity for DNA. The clinical relevance of this mechanistic difference remains to be elucidated. Structurally, mifepristone and onapristone differ in stereochemistry at C_{18}, with onapristone being inverted from the usual steroid nucleus (Figure 5).

Estrogens are produced primarily in the ovaries and are responsible for stimulation of development of female sex organs, mammary glands, and various secondary sexual characteristics. Therapeutic uses of estrogen agonists include oral contraception (in combination with progestins), hormone replacement therapy in postmenopausal women, and treatment for dysmenorrhea, dysfunctional uterine bleeding, acne, hirsutism, failure of ovarian development, coronary artery disease, osteoporosis, and prostate cancers.

Steroidal estrogens (*e.g.*, 17β-estradiol, estrone, Figure 6) have a characteristic phenolic A ring. Natural estrogens are deactivated in the liver; however, an α-substituent at C_{17} interrupts this metabolism (see

ethinyl estradiol). Several non-steroidal estrogens exist including flavinoids and di- and triphenylethylenes (*e.g.*, diethylstilbestrol).

Currently identified anti-estrogens and partial estrogen agonists are predominantly triphenylethylenes. These include tamoxifen,[72] chlomiphene, keoxifene, and **1** (Figure 6).[73] Steroidal anti-estrogens have been prepared and contain a long side chain at C_7 (*e.g.*, ICI 164,384 and ICI 182,780).[74] Breast cancer is the current predominant therapeutic use for estrogen antagonists. Recent molecular insights into the transcriptional transactivating functions of estrogen receptor and other steroid receptors have opened new avenues for drug discovery, making possible the identification of compounds that demonstrate selectivity for a desired biological response.

The estrogen receptor has two distinct regions that confer transactivation of transcription: TAF-1 and TAF-2. Mutant forms of human ER (hER) have been constructed in which either TAF-1 or TAF-2 is genetically "excised". Cotransfection into mammalian cells of TAF-deleted hER genes or wild-type hER cDNAs, together with a plasmid containing an estrogen-responsive reporter gene such as luciferase cDNA, allows the rigorous analysis of the role played by TAF-1 and TAF-2 in the activation of transcription of various target genes by the ER. Using this cotransfection assay, it is possible to dissect the influences on ER-driven transcription of (i) cell background, (ii) promoter context, and (iii) activating ligand (various hER agonists, antagonists, and partial agonists).

When a particular ligand (*e.g.*, estradiol, tamoxifen, or ICI 164,384) interacts with the ER, it induces (or stabilizes) a particular conformation of the receptor. Full agonists, such as 17β-estradiol, induce a "fully active" conformation, in which both TAF-1 and TAF-2 are "exposed" and active. Full antagonists, such as ICI 164,384, appear to bind to ER (competitively with estradiol) and expose neither TAF-1 nor TAF-2. Partial agonists drive the ER into conformations "intermediate" between the fully active and fully inactive conformations driven by estradiol and ICI 164,384, respectively.

Interestingly, not all partial agonists drive the receptor into the same conformation. Tamoxifen appears to induce "exposure" of TAF-1 but not of TAF-2. Tamoxifen therefore functions as an agonist for TAF-1-dependent functions and as an antagonist for TAF-2-dependent functions. The former appears to underlie its estrogen-mimetic pharmacological effects in uterine tissue,[75] and the latter appears to account for its estrogen-blocking effects in breast cancer. Other compounds drive equally reproducible, partially-activating conformations of the ER, differing from that driven by tamoxifen. With the appropriate partial agonists for the ER, therefore, it is possible to achieve transcriptional enhancement of only a subset of estrogen-responsive genes. Compounds with a subset of estrogen's full spectrum of activities can be identified using assays based upon these principles.

Once exposed as a consequence of a ligand-induced conformational change in the ER, TAF-1 and TAF-2 can each independently enhance transcription of some estrogen-responsible genes, presumably by interacting with specific intracellular partner proteins. The recent identification of a 160 kDa estrogen receptor-associated protein (ERAP160)[76] lends further credence to this model. ERAP160 binds to ER through interactions

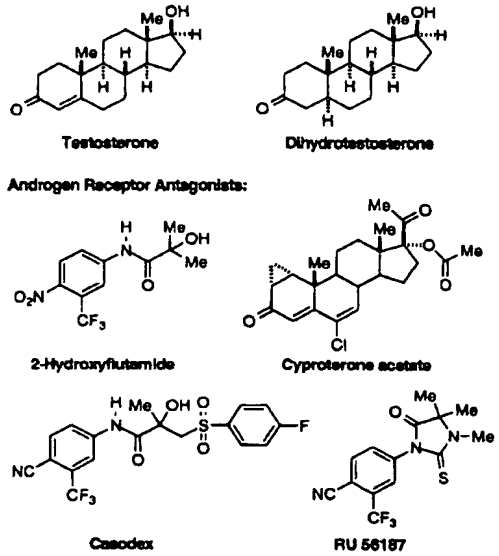


Figure 7. Androgen receptor agonists and antagonists.

involving TAF-2 in an estradiol-dependent manner. The binding of anti-estrogens to the receptor blocks the binding of ERAP160. It has been suggested that ERAP160 mediates transactivation of ER and that the ability of anti-estrogens to block ER-ERAP160 complex formation may account for their therapeutic effects in breast cancer. Although the identities of other postulated partner proteins remain unknown, indirect evidence indicates that their expression varies from cell type to cell type. The identification of these postulated partner proteins will provide yet another attractive target for the development of highly selective small-molecule drugs.

Emerging evidence supports the utility of an approach similar to that taken with ER and PR for the development of tissue-selective partial agonists for other receptors including PR, AR, MR, GR, and VDR.

Androgens are synthesized in the testes, adrenal cortex, and ovaries. The net effect of endogenous androgens reflects the combined actions of the secreted hormone, testosterone (Figure 7); its 5α-reduced metabolite, dihydrotestosterone; and its estrogenic derivative, estradiol. Androgens serve different functions at different stages of male development and have clear therapeutic uses in the treatment of hypogonadism, growth retardation, breast carcinoma, and osteoporosis. The actions of androgens are mediated through AR.[21-23] Compounds that block the action or synthesis of androgens have proven useful in treatment of diseases such as prostate cancer, prostatic hypertrophy, hirutism, male pattern baldness, and acne. Among the most potent orally active anti-androgens (Figure 7) is cyproterone acetate. This compound possesses progestational activity and suppresses the secretion of gonadotrophins, both of which are unwanted side effects. Other anti-androgens include flutamide, a prodrug for the active metabolite, 2-hydroxyflutamide,[77] casodex,[78] and an analogue of nilutamide.[79]

Glucocorticoids and mineralocorticoids are steroid hormones produced by the adrenal cortex. Glucocorti-

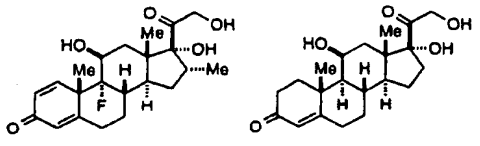


Figure 8. Glucocorticoid receptor agonists.

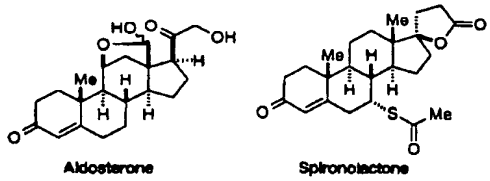


Figure 9. Mineralocorticoid receptor modulators.

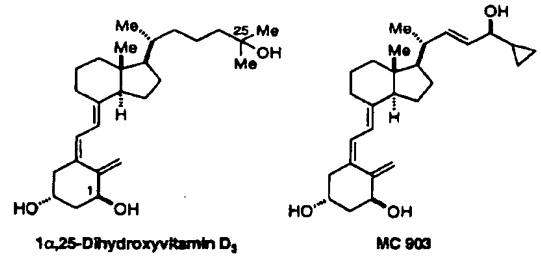


Figure 10. Vitamin D receptor agonists.

coids influence almost every organ and tissue in the body, affecting responses as diverse as behavior, immune function, and carbohydrate metabolism. Cortisol is the most potent naturally occurring glucocorticoid and stimulates or inhibits various biological functions. Synthetic glucocorticoid agonists can be divided into two groups: (i) 4-pregnene or 4-androstenc derivatives and (ii) derivatives of cortisol or dexamethasone (Figure 8). It is not surprising, when the many actions of the glucocorticoids are considered, that these compounds actually have mixed agonist–antagonist activities when examined *in vivo*.

Mineralocorticoids (Figure 9) such as aldosterone regulate electrolyte balance in the kidneys, salivary glands, sweat glands, and gastrointestinal tract. Aldosterone acts by altering the ATP-dependent membrane transport of sodium and potassium ions. The action of aldosterone is inhibited by spironolactone and progesterone. Both act as competitive inhibitors of aldosterone by forming ligand–receptor complexes that are inactive.

A vitamin D_3 precursor is photochemically synthesized in the skin from 7-dehydrocholesterol and then undergoes a hydrogen shift to become vitamin D_3. As with hormones secreted by endocrine glands, this product is transported in the blood to distal sites where it is metabolized to the hormonally active form, calcitriol (1α,25-dihydroxyvitamin D_3, Figure 10), which then affects target tissues through interaction with the VDR, eventually resulting in increased plasma calcium concentrations. The conversion of vitamin D_3 to calcitriol occurring in the kidney is regulated by a negative-feedback control involving free calcium concentrations in the plasma.

The role of calcitriol in controlling the expression of a broad spectrum of genes is becoming increasingly evident. In addition to its active role in calcium homeostasis, calcitriol regulates genes associated with cell growth and tissue-specific structure. It is also responsible for maintaining the precise control of the concentration of calcium and phosphate ions in the plasma by modulating their absorption from the small intestine, enhancing their mobilization from bone and altering their excretion via the kidney. A growing body of evidence indicates that calcitriol also plays a role in the control of proliferation and differentiation of several cell types including epidermal keratinocytes. This effect of VDR agonists on skin has been utilized clinically in the treatment of psoriasis. Vitamin D analogues are also capable of causing differentiation of malignant cells, driving interest in VDR agonists in the treatment of leukemias and breast cancer.

All of the genomic effects of calcitriol are mediated by the VDR, which has been characterized biochemically from a number of tissues derived from many different animal species. There is evidence that certain rapid effects of vitamin D may reflect direct non-VDR-mediated actions at the plasma membrane. One compound that displays tissue-selective VDR agonist action is MC 903 (Figure 10),[80] which mimics the effects of vitamin D_3 on skin without increasing plasma calcium concentrations. It is not presently clear to what extent the tissue-selective actions of compounds such as MC 903 reflect intrinsic pharmacodynamics or pharmacokinetics or differential drug distribution. There is potential for other such tissue-specific vitamin D_3 partial mimics in the treatment of various skin diseases and cancers.

II. Intracellular Receptor Subtype Selective Compounds. Vitamin A (retinol) is derived exclusively from the diet as preformed retinol, retinyl esters, or carotenoids (provitamin A) and is stored primarily in liver as retinyl esters. Like vitamin D_3, retinol is transformed in the body to a variety of active metabolites that play important roles in several diverse cellular processes, including embryonic development, vision, reproduction, bone formation, hematopoiesis, metabolism, cellular differentiation, cellular proliferation, and programmed cell death.[81]

Retinal (vitamin A aldehyde) is required for retinal function. Other vitamin A derivatives, including *all-trans*-retinoic acid (ATRA or vitamin A acid), play an essential role in growth and differentiation of epithelial tissue and are necessary for reproduction, embryonic development, and bone growth. These actions of ATRA and related retinoic acid isomers (i.e., 9-*cis*-RA, discussed below) are mediated by RA IRs, which regulate gene expression.[82]

The profound effect s of retinol metabolites on cellular differentiation and proliferation have spurred the synthesis of thousands of RA analogues (retinoids) with potential use in a variety of skin disorders and malignant disease. Presently, the naturally occurring retinoids (Figure 11) ATRA (an active hormone) and 13-*cis*-RA (most likely acting by giving rise to ATRA and possibly 9-*cis*-Ra) are used for the treatment of severe acne, while synthetic etretinate is prescribed for severe, refractory psoriasis. More recently, ATRA and 13-*cis*-RA have shown promise in the control of cancers or precancers such as acute promyelocytic leukemia,[83] head

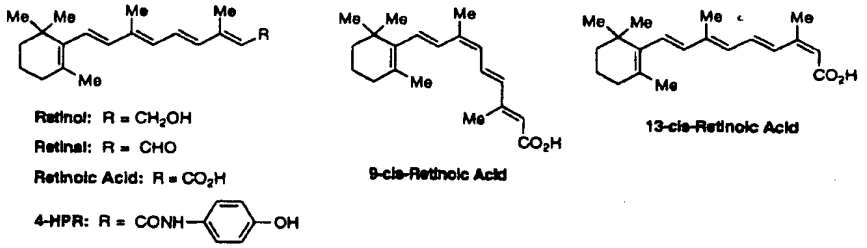


Figure 11. Retinoic acid and derivatives.

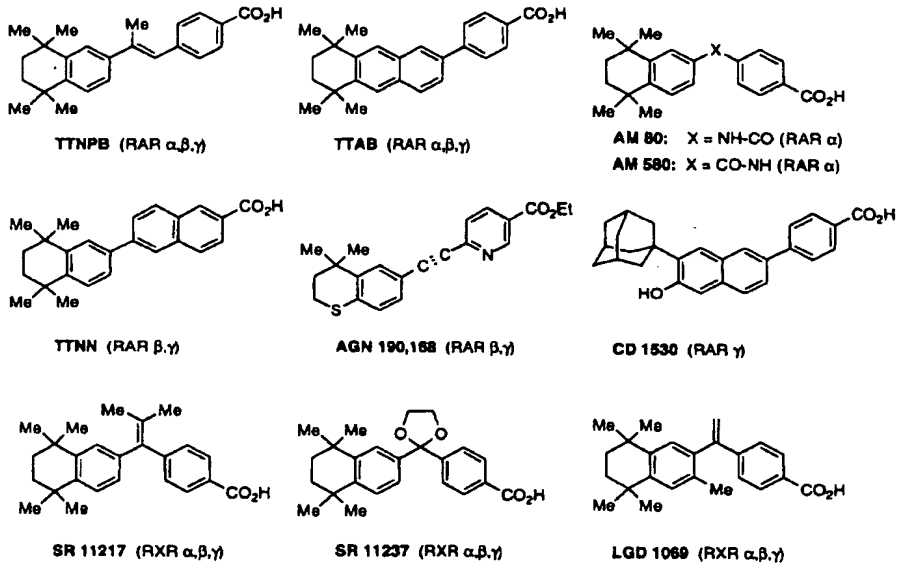


Figure 12. Synthetic retinoids.

and neck cancer,[84] and cervical dysplasia,[85] either as single agents or in combination with other agents such as interferon α. Other synthetic retinoids are in various stages of development for treatment of cancer and skin diseases, including *N*-(4-hydroxyphenyl)retinamide (4-HPR), which is in phase II trials as a chemopreventive agent in breast cancer treatment.[86] Retinamide is likely a prodrug for ATRA.

Unfortunately, widespread clinical use of the currently available retinoids is limited by undesirable side effects. These include mucocutaneous irritation, elevations in plasma triglycerides, headache, bone toxicity, and teratogenicity. The many diverse actions of retinoids, both desirable and undesirable, arise through activation of multiple retinoid receptor subtypes; thus retinoids with receptor subtype selectivity may have improved therapeutic indices.

To date six IR subtypes (or isoforms) that can be activated by ATRA in cells have been identified.[82] Each receptor is encoded by a separate gene. Three of these, RARα, RARβ, and RARγ, are close genetic homologues. ATRA binds directly to each of the RARs, leading to activation of the RARs as transcription factors. The remaining three IRs responsive in cell culture to ATRA are members of the retinoid X receptor subfamily and are designated RXRα, RXRβ, and RXRγ. The RXRs are close genetic homologues of each other, but are less closely related to the RARs. Although the RXRs can be activated by ATRA in living cells, ATRA does not bind to the RXRs directly. ATRA activates RXRs indirectly upon conversion to 9-*cis* RA, the endogenous ligand for the RXRs, which binds to and activates both RXRs and RARs.[87] The pharmacological effects of 9-*cis*-RA, the first novel non-peptidyl hormone described since vitamin D_3 was discovered in 1968, imply possible utility in the treatment of cancer and skin diseases. Chemically synthesized 9-*cis*-RA (LGD1057) is currently in clinical trials in oral and topical formulations for cancer indications.

In addition to endogenous retinoids such as ATRA, 9-*cis*-retinoic acid and 13-*cis*-RA, synthetic, non-natural retinoids with novel retinoid receptor subtype selectivity are emerging as potentially exciting drugs. Investigators have used receptor binding and cotransfection assays to characterize known synthetic retinoids and newer analogues (Figure 12). Highly potent retinoids such as TTNPB and TTAB selectively activate the RAR subfamily but do not effectively distinguish among the isoforms.[88] Both AM-80 and AM-580 display selectivity for the RARα isoform,[89] while TTNN is representative of compounds selective for the RARβ and RARγ subtypes.[88,89] Phase III clinical trials for topical treatment of acne and psoriasis have been completed using AGN 190,168, a novel RARβ- and RARγ-selective compound.[90]

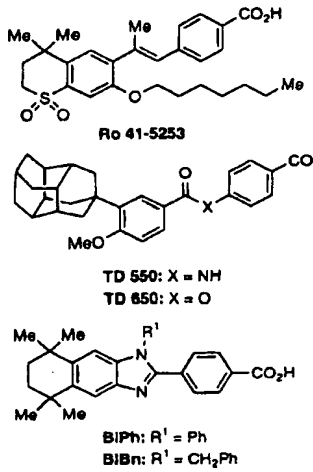


Figure 13. RAR antagonists.

CD 1530 is representative of structures reported to selectively activate the RARγ subtype.[91]

Within the past 2 years, novel classes of RXR-selective retinoids have also been described. Both SR11217 and SR11237 display activation profiles for RXRα similar to that of 9-*cis*-RA at 100 nM.[92] Neither compound activates RAR subtypes. Recently, a potent series of RXR-selective retinoids was developed by capitalizing on the observation that incorporation of a methyl substituent at the 3-position of the tetrahydronaphthalene moiety of TTNPB results in a retinoid with modest activity at all RAR and RXR subtypes.[93] A potent member of this new class, LGD1069, represents the first RXR-selective synthetic retinoid to enter clinical trials for the treatment of cancer.

Several RAR-selective antagonists have also recently been described. A series of sulfone derivatives related to Ro 41-5253 exhibits selectivity for the RARα subtype (Figure 13).[94] Ro 41-5253 was shown to antagonize the teratogenic effects of the RARα-selective agonist AM-580 in rat limb bud cell cultures and in mice. Additional RAR antagonists include TD550, TD650, BIPh, and BIBn, which inhibit retinoid-induced differentiation of human promyelocytic leukemia HL60 cells.[95,96] As yet, no RXR-selective antagonists have been reported.

Data now emerging support separable biological roles for the various RAR and RXR subfamilies and individual subtypes in the control of cell proliferation, differentiation, and programmed cell death (apoptosis). RAR-selective compounds are sufficient to stimulate replication of human cytomegalovirus (hCMV) and induce the differentiation of an embryonal cell line that supports the growth of hCMV.[97] Further analysis of the pharmacological actions of the retinoids and identification of more selective analogues, in conjunction with studies using molecular and cellular biological approaches, are driving elucidation of the biological roles of the retinoid receptor subtypes and the delineation of the potential therapeutical uses of receptor subtype selective retinoids. Additional synthetic retinoids with useful patterns of selective interaction with the RAR and RXR subtypes hold great promise as pharmacological tools for biological investigations and as novel pharmaceuticals.

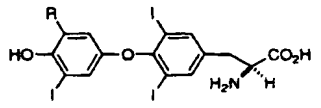


Figure 14. Thyroid hormones.

The thyroid gland is the source of two different thyroid hormones. Thyroxine (T_4) and triiodothyronine (T_3)[98] (Figure 14) are essential for normal growth and development and play an important role in controlling energy metabolism. Changes in the cardiovascular system are prominent consequences of the action of thyroid hormones. Stimulation of cholesterol metabolism to bile acids and lower plasma cholesterol levels result from elevated levels of thyroid hormones. A great many structural analogues of thyroxine have been synthesized in order to define structure–activity relationships, detect antagonists of thyroid hormones, or find compounds exhibiting desirable activity with reduced unwanted side effects.

Three TR subtypes, TRα1, TRα2, and TRβ1, have been identified in human tissues.[99] A fourth receptor subtype, TRβ2, was subsequently isolated from the rat[100] and has been identified in human tissues.[101] The expression of the different TR subtypes is regulated both transcriptionally and post-transcriptionally. Each messenger RNA encoding a TR subtype shows characteristic patterns of developmental, tissue-specific, and hormonal regulation. The complexity of the TRs and their differing patterns of expression suggest that some of the myriad actions of thyroid hormone are mediated by specific TR subtypes. Such specific physiological roles of TR subtypes would imply better defined pharmacological effects for thyroid hormone agonists with TR subtype selectivity.

For other hormones of medical importance, including glucocorticoids and estrogens, receptor subtypes have not yet been identified, although in some senses MR can be thought of as a GR subtype at sites outside the kidney. Among the many orphan receptors (see below) and novel IRs that continue to be identified, currently unrecognized IR subtypes for known hormones may await identification.

III. Orphan Receptors. The ongoing discovery of orphan IRs continues to expand the list of superfamily members. The various orphan receptors are likely to play important functional roles, since (i) their sequences are highly conserved in mammals and even between phyla, (ii) they often have restricted spatial and temporal patterns of expression, (iii) transgenic animals in which various orphan receptors have been "knocked out" show functional impairment or lethality, and (iv) at least some of the orphan IRs can be implicated in the control of specific promoters (for example, hepatic nuclear factor-4 [HNF-4]). For the most part, the functional roles of these orphan IRs *in vivo* remain unknown. It is likely that some of these orphan receptors represent the signal-transducing receptors for currently uncharacterized endocrine, paracrine, or intracrine hormonal regulatory systems. Other orphans IRs may represent subtypes of receptors for known ligands. Thus the RXRs (originally considered orphans) bind and are activated by 9-*cis*-RA.[87]

The restricted tissue distribution of some orphan IRs and the responses of these orphan receptors to known pharmacophores invite speculation about their possible functions. For example, orphan IRs, including chicken ovalbumin upstream promoter transcription factor (COUP-TF),[102] COUP-TFβ,[103] and HNF-4,[104] and the three peroxisome proliferator activated receptor (PPAR) subtypes (PPARα, PPARβ or NUC1, PPARγ),[105] appear to be involved in the control of lipid, cholesterol, or lipoprotein metabolism, rendering them interesting from a pharmaceutical perspective. Known xenobiotics and drugs, such as the fibrate antihyperlipidemics, which induce peroxisome proliferation, appear to act through PPARs. Other orphan receptors, *e.g.*, nerve growth factor induced-B (NGFI-B)[106,107] and the related Nur77 are expressed after cellular exposure to NGF or other growth factors and appear to influence cellular susceptibility to apoptosis. Although their function remains to be elucidated, the thyroid-related (TR2) orphan IR[101] and its splice variants are expressed in a pattern essentially limited to the tissues of the genitourinary tract. Orphan IRs and other members of the superfamily also exhibit overlapping tissue and developmental distribution together with overlapping specificity for response elements within target genes. In these instances expression of target genes in a given tissue mauy be determined by interaction between members of the superfamily receptor complement present in that tissue.

Additional complexity is added with the identification of a growing number of IR subfamily members, as exemplified by the retinoid receptor family. The RXR subfamily was the first retinoid-related subfamily to be identified. Members of this subfamily form heterodimers not only with the retinoid-related RARs, but also with other hormone-activated and orphan IRs including VDR, TR, and PPARs. Recently, the RAR-related orphan receptors (RORs)[108] and retinoid Z-related receptors (RZRs)[109] were identified. The RORs share common DNA- and putative ligand-binding domains, but differ in the N-terminal domains that are generated by alternative RNA splicing. Different members of this subfamily show different binding affinity for the RAR-related orphan response element (RORE) and, as a result, are able to mediate both constitutive and low-level transcription activation of target genes. RZRα and -β, although sharing a high degree of homology, have a different tissue distribution, with expression of RZRβ confined to brain tissue. The RZRs bind as monomers to natural and artificial retinoic acid response elements containing hexameric half-sites and are also able to form homodimers on selected response elements.

Lastly, there are orphan IRs that appear to be constitutively active in the absence of added ligands. These receptors may actually be responding to "intracrine" small-molecule ligands such as metabolic intermediates, *e.g.*, certain fatty acids to which PPARs respond. It is not necessarily the case that all orphan IRs have activating endogenous ligands. For example receptors such as COUP-TF and PR (in some species) can be activated by phosphorylation of appropriate residues by protein kinase A;[110] the physiological and pharmacological relevance of such phosphorylation in modulation of IR activity remains to be definitively established. In any event the orphan IRs potentially are novel targets for pharmaceutical intervention. The actualization of the potential inherent in the orphan IRs is a major challenge in IR-related drug discovery.

STATs and Drug Discovery. In addition to the steroid and small molecule hormones that control gene expression through interactions with IRs, there are peptide and protein ligands in the systemic circulation that produce alterations in gene expression in their target cells to which they bind. Included in this class of proteins are the cytokines (*e.g.*, the interferons and interleukins) and growth factors (*e.g.*, epidermal growth factor). The modulatory proteins are collectively termed extracellular signaling proteins (ESPs).[4] ESPs cannot readily enter cells; they act by binding at the cell surface to specific receptors that span the cell membrane. Thus they can indirectly initiate a chain of events (characterized only recently for a growing number of ESPs) that culminate in changes in the pattern of cellular gene expression. The exact changes elicited are characteristic for the inciting ESP stimulus. For example, after a cell is exposed to interferon-α, specific genes are expressed yielding proteins that (i) render the cell more resistant to viral infection and (ii) reduce the rate at which the cell proliferates. These specific changes in gene expression following exposure of cells to ESPs are in many cases mediated by members of a newly discovered transcription factor superfamily called STATs.[4]

The STATs characterized to date range in molecular mass from roughly 80 to 113 kDa and are not genetic homologues of any other known group of proteins. Among the ESPs, which at least in part appear to exert their effects on cells through specific STATs,[111] are the interferons (IFNs) [IFNα, -β, and -γ]; the colony-stimulating factors (CSFs) [erythropoietin (Epo), granulocyte colony-stimulating factor (G-CSF), macrophage colony-stimulating factor (M-CSF), and granulocyte macrophage colony-stimulating factor (GM-CSF), and probably the recently described thrombopoietin (Tpo)]; various interleukins (ILs) [IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-9, IL-10, IL-11, IL-12, IL-13]; various growth factors [including epidermal growth factor (EGF), platelet-derived growth factor (PDGF), oncostatin-M (OncoM), leukemia inhibitory factor (LIF), and ciliary neurotrophic factor (CNTF)]; and several peptidyl hormones [*e.g.*, growth hormone (GH) and prolactin].

The identification of the first STATs[4,112] led to the elucidation of the biochemical events that mediate the changes in gene expression in response to interferons. Surprisingly, there is an underlying unity in the way that many additional ESPs, with their myriad of distinctive biological effects, act to control gene expression after binding to their cell surface receptors. In response to an ESP stimulus, specific STAT proteins are phosphorylated on tyrosyl residues within minutes after the binding of the ESP to its cell-surface receptor. This phosphorylation of STAT proteins is mediated by specific kinases called Janus kinases, or JAKs (see below) and results in their conversion from latent to active transcription factors.

To date, six different STAT family members (STATs1, -2, -3, -4, -5, -6) have been discovered, and as shown in Table 2 the cytokines that activate all of these STATs have been identified. However, it is highly likely that additional STATs will be found that participate in signal transduction of still other cytokines.

Table 2. Cytokines Utilizing the JAK/STAT Pathway

cytokine	STAT activation	JAK activation[a]
IFNα	STAT1, -2	JAK1, Tyk2
IFNγ	STAT1	JAK1, JAK2
IL-10	STAT1, -3	JAK1, Tyk2
IL-2	STAT5, -3	JAK1, -3
IL-7	STAT5, -3	JAK1, -3
IL-9	STAT5, -3	JAK1, -3
IL-15	STAT5, -3	JAK1, -3
IL-4	STAT6	JAK1, -3
IL-13	STAT6	JAK1
IL-3	STAT5	JAK2
IL-5	STAT1, -3	JAK2
GM-CSF	STAT5	JAK2
IL-6	STAT1, -3	JAK1, -2, Tyk2
IL-11	STAT3	JAK1, -2, Tyk2
LIF	STAT3	JAK1, -2, Tyk2
OSM	STAT1, -3	JAK1, -2, Tyk2
CNTF	STAT3	JAK1, -2, Tyk2
IL-12	STAT3, -4	Tyk2, JAK2
G-CSF	STAT3, -5	JAK1, -2
Epo	STAT5	JAK2
Tpo	STAT5	JAK2
prolactin	STAT5	JAK2
growth hormone	STAT1, -3, -5	JAK2
CSF-1/M-CSF	STAT1, -3	?
EGF	STAT1, -3, -5	JAK1
PDGF	STAT1, -3	?

[a] A question mark (?) indicates that JAK activation has not been reported.

The colony-stimulating factors and interleukin-3 promote the growth of specific cell lineages within the bone marrow, giving rise to the mature cell types found in the blood. GM-CSF and G-CSF are currently used following cancer chemotherapy to increase the speed with which the white blood cell counts return to levels that are protective against infection or to facilitate the process of bone marrow transplantation in cancer patients. Epo has been extraordinarily successful in treatment of anemia due to renal failure. Interferon-β is used in the management of relapsing multiple sclerosis. Interferon-α is one of the cytokines produced by cells in response to viral infection. Recombinant IFN-α has been successfully utilized in the treatment of infectious hepatitis, hairy cell leukemia, and other cancers. The clinical utility of a variety of other cytokines is currently being assessed. The definition of the mechanisms by which the JAKs and STATs are activated and an understanding of their role in ESP signal transduction present new opportunities to discover orally bioavailable small molecule drugs mimicking or blocking medically important ESPs.

Mechanism of JAK/STAT-Mediated Signal Transduction

After an ESP binds to its cognate receptor, a cascade of events is initiated that leads to modulation of gene expression. The primary driver of this cascade appears to be protein phosphorylation. Receptor occupancy, probably through receptor dimerization, leads to changes in the cytoplasmic domain of the receptor that are "recognized" intracellularly. Evidence indicates that the altered receptor cytoplasmic domain becomes an effective "docking platform" for the appropriate members of a tyrosine protein kinase family known as the "Janus kinase" or JAK family[113-115] and/or members of the STAT family of latent transcription factor subunits. Assembly of an appropriate complex of JAKs and STATs anchored to the cytoplasmic domain of various ESP receptors results in tyrosyl phosphorylation and biochemical activation of particular JAKs. The activated JAKs then phosphorylate a subset of STAT proteins at a specific tyrosyl residue.

Although some receptors that utilize the JAK/STAT signaling pathway have intrinsic tyrosyl kinase activity (*e.g.*, receptors for EGF and PDGF), most of these receptors do not. Furthermore all STAT phosphorylation is thought to be dependent upon the JAK kinases that associate noncovalently with the cytoplasmic domains of various ESP receptors. The receptors for ESPs are diverse and, with the exception of those for tumor necrosis factor (TNF) and IL-1, all seem to couple to the STAT signaling pathway through JAK activation. Receptors that bind ESPs as single chains (*e.g.*, receptors for growth hormone, Epo, prolactin, and G-CSF) dimerize after ESP binding. This dimerization appears to lead to localization and activation of particular JAKs. Receptor multimerization also appears to be involved in JAK activation by ESPs that bind receptors with multiple chains. The receptors for one group of cytokines (IL-3, GM-CSF, and IL-5) are formed from different α chains and a common β chain. JAKs interact with the β chain. Lastly, there are cytokines (IL-6, LIF, OncoM, and CNTF) whose receptors are composed of specific α chains and a common protein component termed gp130. The action of these ESPs depends upon oligomerization of the gp130 subunit, which associates with specific JAKs, to activate the STAT signaling pathway.

Tyrosyl-phosphorylated STATs assemble into multimeric complexes, apparently stabilized by intermolecular interactions between src homology 2 (SH2) domains[112,116] and phosphorylated tyrosyl residues within the STATs. SH2 domains, originally defined based on the src oncogene, are involved in binding to phosphotyrosine residues. A central region in the SH2 domain that includes the arginine residue that directly binds to phosphotyrosine is completely conserved among the STAT proteins. Different specific STAT complexes appear to be induced in response to different ESPs. These STAT-containing complexes move from the cytoplasm into the nucleus. Once in the nucleus, activated STAT complexes bind to specific response elements in the promoters of genes responsive to that ESP, acting as biochemically active transcription factors. Following ESP activation of JAK/STAT signaling, the cascade of events appears to be negatively regulated by the action of protein tyrosyl phosphate phosphatases. An important role for protein tyrosyl phosphate phosphatases in limiting ESP-mediated responses is implied by the ability of the protein phosphatases inhibitor vanadate to activate JAK/STAT-mediated transcription.[117]

JAK/STAT-mediated changes in the pattern of gene expression lead to alterations in levels of corresponding encoded proteins and therefore to altered cell function. As with hormone-induced IR-mediated changes in gene expression, the consequences of STAT signal transduction show a gradual onset (minutes to hours) and can last a relatively long time (hours to days). The STATs therefore exert their activity in a manner analogous to that of the IRs: the STATs transduce ESP signals that

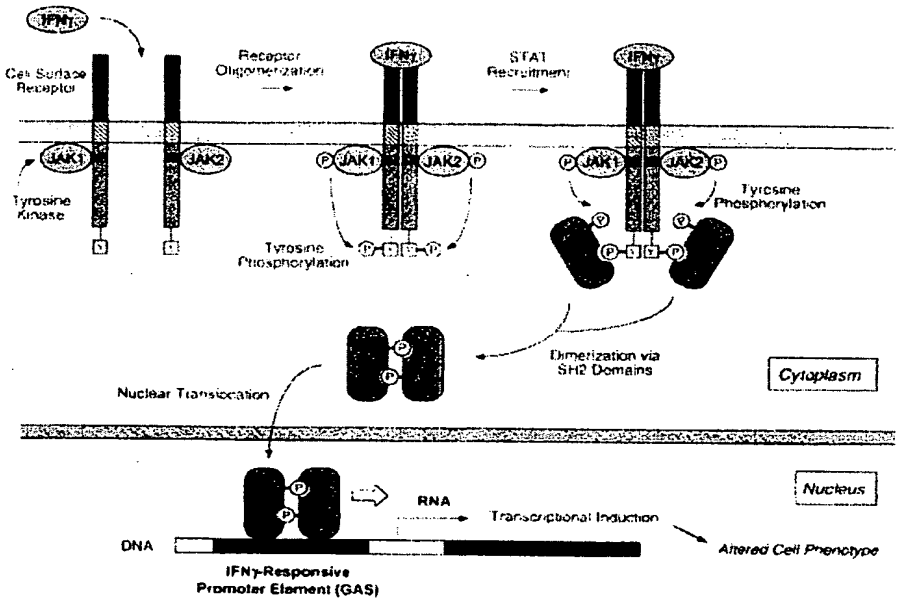


Figure 15. The signal transduction pathway utilized by interferon-γ (IFN-γ). Binding of IFN-γ to the IFN-γ receptor causes multimerization of the receptor and activation of JAK1 and JAK2. These activated JAKs phosphorylate STAT1. This leads to formation of the active transcription factor. After entering the nucleus, the STAT1 homodimer recognizes the IFN-γ activation sequence (GAS) in the promoters of IFN responsive genes and enhances transcription of those genes.

control gene expression, and the IRs serve a similar role for the non-peptidyl hormones.

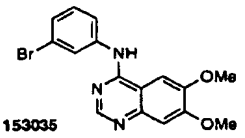
The signal transduction pathway utilized by IFN-γ is shown in Figure 15. As described above, the first step in IFN-γ signal transduction is the binding of IFN-γ to its cell surface receptor. Receptor binding results in receptor oligomerization (a simplified depiction is shown in Figure 15), leading to activation of the receptor-associated JAKs, presumably by cross-phosphorylation. The receptor cytoplasmic domain is then in turn phosphorylated on tyrosine, presumably by the JAKs themselves. A single phosphorylated tyrosine residue on the receptor serves as a docking site for STAT1 (via its SH2 domain); STAT1 then becomes phosphorylated on a specific tyrosyl residue, again presumably via the JAKs.[118] The phosphorylation of this STAT leads to its dimerization, yielding an active transcription factor. The STAT1 dimer can then move into the nucleus and bind to a DNA sequence element, known as an IFN-γ activation sequence (GAS), in the promoter of IFN-γ responsive genes. Binding of the STAT1 dimer to the promoter of these genes causes transcriptional activation in a manner analagous to the IRs. Although IFN-α-induced signal transduction is very similar to that of IFN-γ, the STATs activated are different as is the makeup of the DNA binding complex. Thus, IFN-α treatment leads to activation of JAK1 and tyk2 and phosphorylation of STAT1 and STAT2. Unlike IFN-γ and most other cytokines, the IFN-α-induced multimeric complex, termed interferon-stimulated gene factor-3 (ISGF3)[4,119] also includes a DNA-binding protein, termed p48, that is not a STAT family member. In the absence of the activated STAT components, p48 shows only weak DNA binding. The ISGF3–STAT complex moves into the nucleus, specifically recognizes IFN-α stimulated response elements (ISREs) within the promoters of genes responsive to IFN-α, and enhances the transcription of those genes.

The STATs and JAKs implicated in signal transduction of a variety of ESPs are shown in Table 2. Although there is significant overlap in the STATs participating in complexes induced by different ESPs, it is believed that selective action of a single ESP is obtained based upon the STATs and accessory proteins such as p48 forming the active complex, the overall transcription factor pool within the specific cell type, the precise nature of the STAT response element,[120] and the promoter context in which the STAT response element resides.

Discovery of Drugs Modulating ESP Action

Administration of specific ESPs (*e.g.*, Epo, G-CSF, GM-CSF), various interferons, or IL-2 can have a medically beneficial effect. The therapeutically useful ESPs are often relatively difficult and expensive to manufacture, and they must be administered parenterally and frequently. In some pathological conditions, it may be highly desirable to specifically inhibit the actions of individual ESPs. Currently there are no small-molecule drugs known to act by directly modulating ESP-induced JAK/STAT-mediated signal transduction. However, the known physiological and pharmacological activities of many of the ESPs and their putative roles in the pathophysiology of various diseases suggest possible utilities for small-molecule ESP antagonists. Examples include possible use of interleukin or interferon antagonists in the treatment of inflammation or CSF inhibitors in treatment of leukemias.

A variety of assays can be considered in the construction of screens for compounds capable of modulating ESP-signaling pathways. Among these is a cellular



PD 153035

Figure 16. Inhibitor of epidermal growth factor receptor tyrosine kinase.

transcriptional assay similar to that used for intracellular drug discovery. In this approach a cellular background that harbors the required components of the signaling pathway could be used (*e.g.*, ESP receptor, JAKs, STATs, and tyrosine kinase phosphatases); alternatively, plasmid constructs capable of expressing one or more of the components of the signaling pathway (*e.g.*, ESP receptors, JAKs, or STAT components) can be introduced into the cell. A plasmid consisting of a promoter containing a STAT response element controlling transcription of a reporter gene such as luciferase is then introduced into the cell. Alteration in reporter gene transcription is then used to monitor the efficacy and potency of compounds tested in the screen.

Second, enzymatic assays for selected kinases and phosphatases can be established to identify compounds[121] capable of directly affecting the activity of these critical components of the signaling pathway. In this regard, a very potent and selective inhibitor of the intrinsic tyrosine kinase activity of the EGF receptor has been identified[122] (Figure 16), suggesting that sufficient structural heterogeneity may exist among tyrosine kinases to allow the discovery of selective and potent kinase inhibitors.

Finally, assays can be established that assess physical interactions of the components of the signaling pathway. These could include the interaction between the ESP receptor and the appropriate JAKs, the receptor and STATs, JAKs, and STATs, and homodimeric or heterodimeric STAT complexes.

Each of these approaches has potential advantages and disadvantages, but all could be used to identify novel small molecules capable of modulating the signal transduction pathway induced by selected ESPs. Drug discovery opportunities in this area are rapidly emerging. Small-molecule agonists could act in a variety of steps in the signaling pathway, including inhibition of phosphatase activity or stabilization of interactions between the various components of the pathway. The evidence cited above demonstrating that vanadate induces transcriptional activation of an ESP response element in the absence of the ESP suggests that JAK/STAT pathways are kept quiescent by the continuous action of protein tyrosyl phosphate phosphatases. Any compound that perturbs the activity or localization of these phosphatases would be expected to activate the pathway. In addition, nuclear protein tyrosyl phosphate phosphatases may be involved in the inactivation of phosphorylated STATs. Compounds that act by stabilizing protein−protein interactions, such as the immune suppressants cyclosporin and FK506 are precedents for the sorts of compounds that might stabilize STAT complexes.

Small-molecule ESP agonists could be therapeutically valuable in a variety of important disease states, for example by replacing (i) Epo for treatment of anemia; (ii) G-CSF, GM-CSF or Tpo as an adjunct to cytotoxic cancer chemotherapy; (iii) IFN-α for induction of an antiviral state to treat infectious hepatitis; (iv) IL-2 or IFN-α for cancer therapy; and (v) growth hormone.

ESP antagonists could also act at several different points in the STAT-signaling pathway. These include binding to the cell-surface receptor, disrupting physical interactions that lead to receptor dimerization, preventing interactions between JAK and STAT molecules, interfering with interaction between JAK/STAT complexes and the general transcription complex, and specifically inhibiting JAK activity.

Antagonists of ESP signaling could be useful in inflammatory disease exacerbated by a variety of ESPs including the interferons IL-2, IL-4, and Il-6. IL-2 antagonists could be used in immunosuppressive therapy for graft rejection while an IL-4 antagonist could reduce allergic symptoms. EGF or PDGF antagonists have potential utility in treatment of growth factor dependent cancers. Thus, multiple drug discovery opportunities are represented by modulation of ESP action through the JAK/STAT signaling pathway.

Future Aspects

The progress made in understanding the mechanisms through which extracellular signals, such as hormones and cytokines, act to effect gene transcription has provided many new and interesting avenues for development of therapeutically important small-molecule drugs. As these signal transduction pathways are dissected further, the importance of receptor conformation, receptor interactions with accessory proteins, the roles of different subclasses of receptors, interactions between different IRs, and the role of orphan receptors in determining the specificity of the action of extracellular signals will become clear. Elucidation will potentially provide still more exciting routes for development of small-molecule drugs tailored to evoke a highly specific response.

Long-term therapy with known IR modulators is associated with detrimental side effects that limit their use. Definition of the consequences of ligand binding, in terms of alteration of receptor conformation, may be the key to overcoming this problem. Alterations in receptor conformation will affect both activation of specific transactivation domains within the receptor and interaction of these domains with other proteins required for transcription initiation. The development of screens that detect specific alterations in receptor conformation is critical to the identification of compounds with selective activity.

One approach is the use of assays that identify compounds capable of activating only a subset of receptor transactivation domains. Identification of cells and/or mutant receptors capable of distinguishing compounds that activate via specific transactivation domains can lead to the discovery of agonists or partial agonists with selective activity. The validity of this approach has been demonstrated for ER and may extend to other IRs. Alternatively, as we gain understanding of the role played by accessory factors in the regulation of transcription, assays designed to directly measure productive interaction between these factors and the IRs may aid in the identification of new classes of selective compounds. Recently, accessory proteins have been identified that associate with a number of different IRs.

These include the estrogen receptor associated proteins (ERAPs),[75] additional ER-associated proteins that are involved in the modulation of ER activity, the triiodothyronine receptor auxiliary protein (TRAP),[123] and the 110 kDa receptor accessory factor (RAF)[124] associated with AR. As these and additional, yet to be discovered, factors are characterized and their tissue distributions are determined, their physiological significance can be elucidated. Both functional and biochemical assays dependent on these proteins can then be established and used to discover compounds with selective therapeutic action.

The growing number of IRs with related subtypes provides another means to discover selective small-molecule modulators. To date, four distinct subfamilities of the retinoid receptor family have been identified: RAR, RXR, ROR, and RZR. Each of these subfamilies contains a variable number of subtypes: three RARs, three RXRs, two RZRs, and two RORs. Subfamily-selective compounds, as well as pan agonists, for the RARs and RXRs have been identified. A variety of assays have demonstrated that retinoid subtype-selective compounds lead to different pharmacologies. By exploiting the existence of subtypes in other IR families, compounds may be found that also exhibit differences in pharmacology. The TR and PPAR receptor families both provide exciting targets with potential utility in the treatment of cardiovascular disease and obesity. Since fibrates, which are currently used to reduce triglycerides, modulate the activity of PPARs, more selective compounds may give cleaner pharmacology. PPARγ is activated during the differentiation of adipocytes; modulating its activity may be important in controlling obesity. Thyroid hormone is clearly implicated in thermogenesis, a critical control point in fat deposition and use. In addition, thyroid hormone deficiency leads to elevated serum lipid levels that can be corrected by replacement therapy. The limited use of thyroid hormone in normal individuals due to its associated side effects may be overcome through the development of receptor-selective compounds.

The interaction between ligand-bound receptors and response elements in target genes may provide another means through which selectivity can be achieved. In general, target genes regulated by binding of the RARs and RXRs contain response elements that consist of two directly repeated half-sites. Recent studies have shown that RXR/RAR heterodimers activate transcription in response to *all-trans*-RA or 9-*cis*-RA by binding to direct repeats separated by five base pairs (termed a DR5 element) such that RAR occupies the downstream half-site. RXR homodimers activate transcription in response to 9-*cis*-RA by binding to direct repeats separated by one base pair (a DR1 element). RAR/RXR heterodimers can also bind to DR1 elements, with greater affinity than the RXR homodimer; however, in most contexts RAR/RXR heterodimers are unable to activate transcription in response to either *all-trans*-RA or 9-*cis*-RA. Thus, RARs appear to inhibit RXR-dependent transcription from these sites. RAR can be switched from a retinoid-dependent activator to an inhibitor when it occupies the upstream half-site of the DR1 element. These findings regarding the interaction between the ligand-bound receptors and their response elements clearly demonstrate that receptor conformation and binding characteristics can be manipulated to alter the physiological outcome of receptor binding.

Not only is tissue distribution of the receptor itself a factor in restricted activity but also the availability of heterodimeric partners is critical in determining receptor activity. The role of heterodimer formation between subfamily members of the same IR family in the regulation of target genes has been demonstrated. A body of evidence now exists indicating that heterodimer formation between different intracellular families also plays an important role in the regulation of gene expression. The receptor complement of a particular cell or tissue type will therefore determine not only the response mediated by a given IR family, but may also significantly affect responses mediated by other intracellular superfamily members. As the role of heterodimer formation between superfamily members is examined, the tools developed will allow us to monitor these interactions, which in turn will enable identification of new drug targets.

Lastly, one of the most promising avenues for selective small-molecule drugs is the identification of modulators of the growing number of orphan receptors. The orphan receptors' tissue distributions and patterns of interactions with other IRs indicate that they play important roles in the regulation of gene transcription. Defining their physiological roles is the first step in exploiting these receptors for drug discovery. Identification of ligands, determination of their effects *in vivo*, and production of transgenic mice in which the gene for the orphan receptor is knocked out or overexpressed are ways that this can be accomplished.

The complexity of signal transduction pathways for IR ligands and for extracellular signaling proteins, such as the cytokines, can lead to the discovery of small-molecule modulators via numerous routes. The future challenge resides in the dissection of these pathways and in determining the optimal points of intercession for useful therapeutic outcome. For the IRs, this will require greater understanding of the proteins that transmit information from the IRs to the general transcription apparatus. A greater understanding of the role of IR phosphorylation may also be useful in the identification of new and useful targets for drug discovery. For the JAK/STAT pathways, further insight into the selective modulation of the activity of specific kinases and phosphatases, as well as the myriad protein−protein and protein−DNA interactions involved in signal transduction will be required. The ability to identify small molecules that modulate these activities in the IR and JAK/STAT pathways depends greatly upon the development of high-throughput screens based upon molecular insights into their mechanisms of action. Although this review describes drug discovery approaches based upon modulation of transcriptional activity, additional targets for modulation of steroid hormone action have been identified, particularly the steroid metabolizing enzymes that are known to both activate and inactivate receptor ligands.

Conclusion

From the perspective of drug discovery, the parallels between IR and STAT signal transduction are clear. Both the STATs and the IRs are latent transcription factors activated by cellular exposure to relevant ligands.

High-throughput, cell-based screens using reporter enzymes can be constructed in which the consequences of transcriptional modulation by potential small molecule agonists of STATs and IRs can be readily assessed. The structures of the reporter plasmids used in the IR and STAT assays are also similar. Each is composed of a reporter enzyme gene under the transcriptional control of a response element in the context of a minimal or naturally responsive promoter. These screens have demonstrated untility in IR drug discovery. The discovery of the JAK/STAT-signaling pathway presents an exciting approach to cytokine-related drug discovery that can yield small-molecule agonists and antagonists with patentability, oral bioavailability, and ease of manufacture. The drug discovery strategies described in this review are designed to identify compounds with novel and therapeutically useful properties.

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References

(1) Hollenberg, S. M.; Weinberger, C.; Ong, E. S.; Cerelli, G.; Oro, A.; Lebo, R.; Thompson, E. B.; Rosenfeld, M. G.; Evans, R. M. Primary Structure and Expression of a Functional Human Glucocorticoid Receptor cDNA. *Nature* **1985**, *318*, 635–641.
(2) Evans, R. M. The steroid and thyroid hormone receptor superfamily. *Science* **1989**, *240*, 889–895.
(3) Amero, S. A.; Kretsinger, R. H.; Moncreif, M. D.; Yamamoto, K. R.; Pearson, W. K. The Origin of Nuclear Hormone Receptor Proteins: A Signal Precursor Distinct from other Transcription Factors. *Mol. Endocrinol.* **1992**, *6*, 3–7.
(4) Schindler, C.; Shuai, K.; Prezioso, V. R.; Darnell, J. E., Jr. Interferon-Dependent Tyrosine Phosphorylation of a Latent Cytoplasmic Transcription Factor. *Science* **1992**, *257*, 809–813.
(5) Buratowski, S. The Basics of Basal Transcription by RNA Polymerase II. *Cell* **1994**, *77*, 1–3.
(6) This notation can be applied to all of the basal transcription factors.
(7) Tijan, R.; Maniatis, T. Transcription activation: A Complex Puzzle with a Few Easy Pieces. *Cell* **1994**, *77*, 5–8.
(8) Pratt, W. B. Interaction of Hsp90 with Steroid Hormone Receptors: Organizing Some Diverse Observations and Presenting the Newest Concepts. *Mol. Cell. Endocrinol.* **1990**, *74*, C69–C76.
(9) Kimura, Y.; Yahara, I.; Lindquist, S. Role of Protein Chaperone YDJ1 in Establishing Hsp90-Mediated Signal Transduction Pathways. *Science* **1995**, *268*, 1362–1365.
(10) Sanchez, E. R.; Hirst, M.; Scherrer, L. C.; Tang, H. Y.; Welsh, M. J.; Jammon, J. M.; Simmons, S. S.; Ringold, G. M.; Pratt, W. B. Hormone-Free Mouse Glucocorticoid Receptors Over Expressed in Chinese Hamster Ovary Cells are Localized to the Nucleus and are Associated with both Hsp 70 and 90. *J. Biol. Chem.* **1990**, *265*, 20123–20130.
(11) Dalman, F. C.; Sturzenbecker, L. J.; Levin, A. A.; Lucas, D. A.; Perdew, G. H.; Petkovitch, M.; Chambon, P.; Grippo, J. F.; Pratt, W. B. Retinoic Acid Receptor Belongs to a Subclass of Nuclear Receptors that do not form "Docking" Complexes with Hsp90. *Biochemistry* **1991**, *30*, 5605–5608.
(12) Allan, G. F.; Leng, K.; Tsai, S.-Y.; Weigel, N. L.; Edwards, D. P.; Tsai, M.-J.; O'Malley, B. W. Hormone and Anti-Hormone Induce Distinct Conformational Changes which are Central to Steroid Receptor Activation. *J. Biol. Chem.* **1992**, *267*, 19513–19520.
(13) Allan, G. F.; Tsai, S.-Y.; O'Malley, B. W.; Tsai, M.-J. Steroid Hormone Receptors and *In Vitro* Transcription. *BioEssays* **1991**, *13*, 73–78.
(14) Naar, A. M.; Boutin, J.-M.; Lipkin, S. M.; Yu, V. C.; Holloway, J. M.; Glass, C. K.; Rosenfeld, M. G. The Orientation and Spacing of Core DNA-Binding Motifs Dictate Selective Transcriptional Responses to Three Nuclear Receptors. *Cell* **1991**, *65*, 1267–1279.
(15) Danielsen, M.; Hinck, L.; Ringold, G. M. Two Amino Acids within the Knuckle of the First Zinc Finger Specify DNA Response Element Activation by the Glucocorticoid Receptor. *Cell* **1989**, *57*, 1131–1138.
(16) Tsai, S. Y.; Carlstedt-Duke, J.; Weigel, N. L.; Dahlman, K.; Gustafsson, J. A.; Tsai, M.-J.; O'Malley, B. W. Molecular Interactions of Steroid Hormone Receptor with its Enhancer Element. *Mol. Endocrinol.* **1988**, *2*, 1221–1229.
(17) Yu, V. C.; Delseret, C.; Anderson, B.; Holloway, J. M.; Devary, O. V.; Naar, A. M.; Kim, S. Y.; Boutin, J. M.; Glass, C. K.; Rosenfeld, M. G. RXRβ: A Coregulator that Enhances Binding of Retinoic Acid, Thyroid Hormone and Vitamin D3 Receptors to their Cognate Response Elements. *Cell* **1991**, *67*, 1251–1266.
(18) Green, G. L.; Gilna, P.; Waterfield, M.; Baker, A.; Hort, Y.; Shine, J. Sequence and Expression of Human Estrogen Receptor Complementary DNA. *Science* **1986**, *231*, 1150–1154.
(19) Conneely, O. M.; Dobson, A. D. W.; Tsai, M.-J.; Beattie, W. G.; Toft, D. O.; Huckaby, C. S.; Zarucki, T.; Schrader, W. T.; O'Malley, B. W. Sequence and Expression of a Functional Chicken Progesterone Receptor. *Mol. Endocrinol.* **1987**, *1*, 517–525.
(20) Misrahi, M.; Atger, M.; d'Auriol, L.; Loosfelt, H.; Meriel, C.; Fridlansky, F.; Guiochon-Mantel, A.; Galibert, F.; Milgrom, E. Complete Amino Acid Sequence of the Human Progesterone Receptor Deduced from Cloned cDNA. *Biochem. Biophys. Res. Commun.* **1987**, *143*, 740–748.
(21) Chang, C.; Kokontis, J.; Liao, S. Molecular Cloning of Human and Rat Complementary DNA Encoding Androgen Receptors. *Science* **1988**, *240*, 324–326.
(22) Lubahn, D. B.; Joseph, D. R.; Sullivan, P. M.; Willard, H. F.; French, F. S.; Wilson, E. M. Cloning of Human Androgen Receptor Complementary DNA and Localization to the X Chromosome. *Science* **1988**, *240*, 327–330.
(23) Lubahn, D. B.; Joseph, D. R.; Sar, M.; Tan, J.; Higgs, H. N.; Larson, R. E.; French, F. S.; Wilson, E. M. The Human Androgen Receptor: Complementary Deoxyribonucleic Acid Cloning, Sequence Analysis and Gene Expression in Prostate. *Mol. Endocrinol.* **1988**, *2*, 1265–1275.
(24) Arriza, J. L.; Weinberger, C.; Cerelli, G.; Glaser, T. M.; Handelin, G. L.; Housman, D. E.; Evans, R. M. Cloning of the Human Mineralocorticoid Receptor Complementary DNA: Structural and Functional Kinship with the Glucocorticoid Receptor. *Science* **1987**, *237*, 268–275.
(25) Weinberger, C.; Thompson, C. C.; Ong, E. S.; Lebo, R.; Gruol, D. J.; Evans, R. M. The c-*erbA* Gene Encodes a Thyroid Hormone Receptor. *Nature* **1986**, *324*, 641–646.

(26) Giguere, V.; Ong, E. S.; Segui, P.; Evans, R. M. Identification of a Receptor for the Morphogen Retinoic Acid. *Nature* **1987**, *330*, 624–629.
(27) Petkovitch, M.; Brand, N. J.; Krust, A.; Chambon, P. A Human Retinoic Acid Receptor Which Belongs to the Family of Nuclear Receptors. *Nature* **1987**, *330*, 444–450.
(28) McDonnell, D. P.; Mangelsdorf, D. J.; Pike, J. W.; Haussler, M. R.; O'Malley, B. W. Molecular Cloning of Complementary DNA Encoding the Avian Receptor for Vitamin D_3. *Science* **1987**, *235*, 1214–1217.
(29) Giguere, V.; Yang, N.; Segui, P.; Evans, R. M. Identification of a New Class of Steroid Hormone Receptors. *Nature* **1988**, *331*, 91–94.
(30) Kliewer, S. A.; Umesomo, K.; Heyman, R. A.; Mangelsdorf, D. J.; Evans, R. M. Retinoid X Receptor Interacts with Nuclear Receptors in Retinoic Acid, Thyroid Hormone and Vitamin D_3 Signaling. *Nature* **1994**, *355*, 446–449.
(31) Hirst, M. A.; Hinck, L.; Danielsen, M.; Ringold, G. M. Discrimination of DNA Response Elements for Thyroid Hormone and Estrogen in Dependent Upon Dimerization of the Receptor DNA Binding Domains. *Proc. Natl. Acad. Sci. U.S.A.* **1992**, *89*, 5527–5531.
(32) Medici, N.; Nigro, V.; Abbondanza, S. C.; Moncharmont, B.; Molinari, A. M.; Puca, G. A. *In Vitro* Binding of the Purified Hormone-Binding Subunit of the Estrogen Receptor to Oligonucleotides Containing Natural or Modified Sequences of an Estrogen-Responsive Element. *Mol. Endocrinol.* **1991**, *5*, 555–563.
(33) Milbrandt, J. Nerve growth Factor Induces a Gene Homologous to the Glucocorticoid Receptor Gene. *Neutron* **1988**, *1*, 183–188.
(34) Hazel, T. G.; Nathans, D.; Lau, L. F. A Gene Inducible by Serum Growth Factors Encodes a Member of the Steroid and Thyroid Hormone Receptor Superfamily. *Proc. Natl. Acad. Sci. U.S.A.* **1988**, *85*, 8444–8448.
(35) Lala, D. S.; Rice, D. A.; Parker, K. L. Steroidogenic Factor 1, a Key Regulator of Steroidogenic Enzyme Expression is the Mouse Homolog of Fushi Tarazu-Factor 1. *Mol. Endocrinol.* **1992**, *6*, 1249–1258.
(36) Beato, M.; Gene Regulation by Steroid Hormones. *Cell* **1989**, *56*, 335–344.
(37) Sone, T.; Scott, R. A.; Hughes, M. R.; Malloy, P. J.; Feldman, D.; O'Malley, B. W.; Pike, J. W. Mutant Vitamin D_3 Receptors which Confer Hereditary Resistance to 1,25-Dihydroxyvitamin D_3 in Humans are Transcriptionally Inactive *In Vitro*. *J. Biol. Chem.* **1989**, *204*, 20230–20234.
(38) Freedman, L. P.; Luisi, B. F.; Korszum, Z. R.; Basavappa, R.; Sigler, P. B.; Yamamoto, K. R. The Function and Structure of the Metal Coordination Sites within the Glucocorticoid Receptor DNA Binding Domain. *Nature* **1988**, *334*, 543–546.
(39) Hard, T.; Kellenbach, E.; Boelens, R.; Maler, B. A.; Dahlman, K.; Freedman, L. P.; Carlstedt-Duke, J.; Yamamoto, K. R.; Gustafsson, J.-A.; Kapstein, R. Solution Structure of the Glucocorticoid Receptor DNA-Binding Domain. *Nature* **1990**, *348*, 458–461.
(40) Schwabe, J. W. R.; Neuhaus, D.; Rhodes, D. Solution Structure of the DNA-Binding Domain of the Estrogen Receptor. *Nature* **1990**, *348*, 458–461.
(41) Luisi, B. F.; Xu, W. X.; Otwinowski, Z.; Freeman, L. P.; Yamamoto, K. R.; Sigler, P. B. Crystallographic Analysis of the Interaction of the Glucocorticoid Receptor With DNA. *Nature* **1991**, *352*, 497–505.
(42) Mader, S.; Kumar, V.; de Verneuil, H.; Chambon, P. Three Amino Acids of the Oestrogen Receptor are Essential to its Ability to Distinguish an Oestrogen from a Glucocorticoid-Responsive Element. *Nature* **1989**, *338*, 271–274.
(43) The terms distal and proximal are made in reference to the amino terminus of the protein.
(44) Umesomo, K.; Evans, R. M. Determinants of Target Gene Specificity for Steroid/Thyroid Hormone Receptors. *Cell* **1989**, *57*, 1139–1146.
(45) Glass, C. K.; Lipkin, S. M.; Devary, O. V.; Rosenfeld, M. G. Positive and Negative Regulation of Gene Transcription by a Retinoic Acid-Thyroid Hormone Receptor Heterodimer. *Cell* **1989**, *59*, 697–708.
(46) Bourguet, W.; Ruff, M.; Chambon, P.; Gronemeyer, H.; Moras, D. Crystal Structure of the Ligand-binding domain of the Human Nuclear Receptor RXR-α. *Nature* **1995**, *375*, 377–382.
(47) Forman, B. M.; Samuels, H. H. Dimerization Among Nuclear Hormone Receptors. *New Biol.* **1990**, *2*, 587–594.
(48) Tsai, S. Y.; Carlstedt-Duke, J.-A.; Weigel, N. L.; Dahlman, K.; Gustafsson, J.-A.; Tsai, M.-J.; O'Malley, B. W. Molecular Interactions of Steroid Hormone Receptor with its Enhancer Element: Evidence for Receptor Dimer Formation. *Cell* **1988**, *55*, 361–369.
(49) Picard, D.; Kumar, V.; Chambon, P.; Yamamoto, K. R. Signal Transduction by Steroid Hormones: Nuclear Localization is Differentially Regulated in Estrogen and Glucocorticoid Receptors. *Cell Regul.* **1992**, *1*, 291–299.
(50) Kumar, V.; Chambon, P. The Estrogen Receptor Binds Tightly to its Response Element as a Ligand-Induced Homodimer. *Cell* **1988**, *55*, 145–156.
(51) Picard, D.; Salser, S. J.; Yamamoto, K. R. A Movable and Regulable Inactivation Function within the Steroid Binding Domain of the Glucocorticoid Receptor. *Cell* **1988**, *54*, 1073–1080.
(52) Landschultz, W. H.; Johnson, P. F.; McKnight, S. L. The Leucine Zipper: a Hypothetical Structure Common to a New Class of DNA Binding Proteins. *Cell* **1990**, *60*, 953–962.
(53) Kumar, V.; Green, S.; Stack, G.; Bery, M.; Jin, J.-R.; Chambon, P. Functional Domains of the Human Estrogen Receptor. *Cell* **1987**, *51*, 941–951.
(54) Hollenberg, S. M.; Evans, R. M. Multiple and Cooperative Transactivation Domains of the Human Glucocorticoid Receptor. *Cell* **1988**, *55*, 899–906.
(55) Tora, L.; White, J.; Brou, C.; Tasset, D.; Webster, N.; Scheer, E.; Chambon, P. The Human Estrogen Receptor has two Independently Non-Acidic Transcriptional Activation Functions. *Cell* **1989**, *59*, 477–487.
(56) Dobson, A. D. W.; Conneely, O. M.; Beattie, W. G.; Maxwell, B. L.; Mak, P.; Tsai, M.-J.; Schrader, W. T.; O'Malley, B. W. Mutational Analysis of the Chicken Progesterone Receptor. *J. Biol. Chem.* **1989**, *264*, 4207–4211.
(57) Nagpal, S.; Friant, S.; Nakshatri, H.; Chambon, P. RARs and RXRs: Evidence for two Autonomous Transactivation Functions (AF-1 and AF-2) and Heterodimerization *In Vivo*. *EMBO J.* **1993**, *12*, 2349–2360.
(58) Berger, T. S.; Parandoosh, Z.; Perry, B. W.; Stein, R. B. Interaction of Glucocorticoid Analogues with the Human Glucocorticoid Receptor. *J. Steroid Biochem. Mol. Biol.* **1992**, *41*, 733–738.
(59) Ojasoo, T.; Raynaud, J.-P. Steroid Hormone Receptors. In *Comprehensive Medicinal Chemistry, Volume 3*; Emmett, J. C., Ed.; Pergamon Press: New York, 1990; pp 1200–1207.
(60) Raynaud, J. P.; Azadian-Boulanger, G.; Ojasoo, T. Expansion Scientifique Francaise. In *Pharmacologie Clinique. Bases de la Thérapeutique;* Giroud, J. P., Mathé, G., Meyniel, G., Eds.; 1988; p 2093.
(61) Horwitz, K. B. Purification, Monoclonal Antibody Produdction and Structural Analyses of Human Progesterone Receptors. *J. Steroid Biochem.* **1988**, *31*, 573–578.
(62) Ilenchuk, T. T.; Walters, M. R. Rat Uterine Progesterone Receptor Analyzed by [^{3}H]R5020 Photoaffinity Labeling: Evidence that the A and B Subunits are not Equimolar. *Endocrinology* **1987**, *120*, 1449–1456.
(63) Vegeto, E.; Shahbaz, M. M.; Wen, D. X.; Goldman, M. E.; O'Malley, B. W.; McDonnell, D. P. Human Progesterone Receptor A Form is a Cell- and Promoter-Specific Repressor of Human Progesterone Receptor B Function. *Mol. Endocrinol.* **1993**, *7*, 1244–1255.
(64) Wen, D. X.; Xu, Y.-F.; Mais, D. E.; Goldman, M. E.; McDonnell, D. P. The A- and B-Isoforms of the Human Progesterone Receptor Operate Through Distinct Signaling Pathways within Target Cells. *Mol. Cell Biol.* **1994**, *14*, 8356–8364.
(65) McDonnell, D. P.; Goldman, M. E. RU486 Exerts Anti-Estrogenic Activities Through a Novel Progesterone Receptor A Form-Mediated Mechanism. *J. Biol. Chem.* **1994**, *269*, 11945–11949.
(66) McDonnell, D. P.; Shahbaz, M. M.; Vegeto, E.; Goldman, M. E. The Human Progesterone Receptor A-Form Functions as a Transcriptional Modulator of Mineralocorticoid Receptor Transcriptional Activity. *J. Steroid Biochem. Mol. Biol.* **1994**, *48*, 425–432.
(67) Teutsch, G.; Philibert, D. History and Perspectives of Anti-Progestins from the Chemist's Point of View. In *Human Reproduction, Vol. 9, Supplement 1;* Edwards, R. G., Ed.; Oxford University Press: Oxford, England, 1994; pp 12–31.
(68) Philibert, D.; Deraedt, R.; Teutsch, G.; Tournemine, C.; Sakiz, E. A New Lead for Steroidal Anti-Hormones. 64th Annual Meeting of the Endocrine Society, San Francisco, 1982, Abstract 668.
(69) Neef, G.; Beier, S.; Elger, W.; Henderson, D.; Wiechert, R. New Steroids with Anti-Progestational and Anti-Glucocorticoid Activities. *Steroids* **1984**, *44*, 349–372.
(70) Kloosterboer, H. J.; Deckers, G. H.; Schoonen, W. G. E. J. Pharmacology of Two New Very Selective Anti-Progestins: Org 31710 and Org 31806. In *Human Reproduction, Vol. 9, Supplement 1;* Edwards, R. G., Ed.; Oxford University Press: Oxford, England, 1994; pp 47–52.
(71) Klein-Hitpass, L.; Cato, A. C. B.; Henderson, D.; Ryffel, G. U. Two Types of Anti-Progestins Identified by their Differential Action in Transcriptionally Active Extracts from T47D Cells. *Nucleic Acids Res.* **1991**, *19*, 303–310.
(72) Wakeling, A. E.; Valcaccia, B.; Newboult, E.; Green, L. R. Non-Steroidal Anti-Estrogens-Receptor Binding and Biological Response in Rat Uterus, Rat Mammary Carcinoma and Human Breast Cancer Cells. *J. Steroid Biochem.* **1984**, *20*, 111–120.

(73) Willson, T. M.; Henke, B. R.; Momtahen, T. M.; Charifson, P. S.; Batchelor, K. W.; Lubahn, D. B.; Moore, L. B.; Oliver, B. B.; Sauls, H. R.; Triantafillou, J. A.; Wolfe, S. G.; Baer, P. G. 3-[4-(1,2-Dipihenylbut-1-enyl)phenyllacrylic acid: a Non-Steroidal Estrogen with Functional Selectivity for Bone over Uterus in Rats. *J. Med. Chem.* **1994**, *37*, 1550−1552.
(74) Wakeling, A. E.; Bowler, J. Steroidal Pure Antiestrogens. *J. Endocrinol.* **1987**, *112*, R7-R10.
(75) Tzukerman, M. T.; Esty, A.; Santiso-Mere, D.; Danielian, P.; Parker, M. G.; Stein, R. B.; Pike, J. W.; McDonnell, D. P. Human Estrogen Receptor Transactivational Capacity is Determined by both Cellular and Promoter Context and Mediated by Two Functionally Distinct Intramolecular Regions. *Mol. Endocrinol.* **1994**, *8*, 21−30.
(76) Halachmi, S.; Marden, E.; Martin, G.; MacKay, H.; Abbondanza, C.; Brown, M. Estrogen Receptor-Associated Proteins: Possible Mediators of Homone-Induced Transcription. *Science* **1994**, *264*, 1455−1458.
(77) Koch, H. Flutamide—a New Non-Steroidal Anti-Androgen. *Drugs Today* **1984**, *20*, 561−574.
(78) Tucker, H.; Crook, J. W.; Chesterson, G. J. Nonsteroidal Antiandrogens. Synthesis and Structure-Activity Relationships of 3-Substituted Derivatives of 2-Hydroxypropionanilides. *J. Med. Chem.* **1988**, *31*, 954−959.
(79) Teutsch, G.; Goubet, F.; Battmann, T.; Bonfils, A.; Bouchoux, F.; Cerede, E.; Gofflo, D.; Gaillard-Kelly, M.; Philibert, D. Non-Steroidal Antiandrogens: Synthesis and Biological Profile of High-Affinity Ligands for the Androgen Receptor. *J. Steroid Biochem. Mol. Biol.* **1994**, *48*, 111−119.
(80) Kragballe, K.; Beck, H. I.; Soggard, H. Improvement of Psoriasis by a Topical Vitamin D_3 Analogue (MC 903) in a Double Blind Study. *Br. J. Dermatol.* **1988**, *119*, 223−230.
(81) Blaner, W. S.; Olson, J. A. Retinol and Retinoic Acid Metabolism. In *The Retinoids: Biology, Chemistry and Medicine*, 2nd Ed.; Sporn, M. B., Roberts, A. B., Goodman, D. S., Eds.; Raven Press: New York, 1994; pp 229−256.
(82) Mangelsdorf, D. J.; Umesono, K.; Evans, R. M. The Retinoid Receptors. In *The Retinoids*; Sporn, M. B., Roberts, A. B., Goodman, D. S., Eds.; Raven Press: New York, 1994; pp 319−349.
(83) Huang, M. E.; Ye, Y. I.; Chen, S. R.; Chai, J. R.; Lu, J. X.; Zhoa, L.; Gu, L. J.; Wang, Z. Y. Use of All-Trans Retinoic Acid in the Treatment of Acute Promyelocyctic Leukemia. *Blood* **1988**, *72*, 567−571.
(84) Hong, W. K.; Lippman, S. M.; Itri, L. M.; Karp, D. D.; Lee, J. S.; Byers, R. M.; Schantz, S. P.; Kramer, A. M.; Lotan, R.; Peters, L. J.; Dimery, I. W.; Brown, B. W.; Goepfert, H. Prevention of Second Primary Tumors with Isotretinoin in Squamous-Cell Carcinoma of the Head and Neck. *N. Engl. J. Med.* **1990**, *323*, 795−801.
(85) Meyskens, F. L.; Surwit, E.; Moon, T. E.; Davis, J.; Alberts, D. S. A Placebo-Controlled Randomized Phase III Trial of Locally Applied β-Trans Retinoic Acid (Vitamin A Acid, RA) in Cervical Intraepithelial Neoplasia (CIN): Favorable Effect On CIN II (Moderate Dysplasia). *Proc. Am. Soc. Clin. Oncol.* **1993**, *12*, 167.
(86) Fomelli, F.; Carsana, R.; Clerici, M.; Cleris, L. Pharmacological Studies on Fenretinide in Breast Cancer Patients and in Rats. In *Retinoids: Progress in Research and Clinical Applications*; Livrea, M. A., Packer, L., Eds.; Marcel Dekker: New York, 1993; pp 473−489.
(87) Heyman, R. A.; Mangelsdorf, D. J.; Dyck, J. A.; Stein, R. B.; Eichele, G.; Evans, R. M.; Thaller, C. 9-*cis*-Retinoic Acid is a High Affinity Ligand for the Retinoid X Receptor. *Cell* **1992**, *68*, 397−406.
(88) Dawson, M. I.; Hobbs, P. D.; Stein, R. B.; Berger, T. S.; Heyman, R. A. Interaction of Retinoids with Retinoic Acid Nuclear Receptor Isoforms. In *Retinoids: Progress in Research and Clinical Applications;* Livrea, M. A., Packer, L., Eds.; Marcel Dekker: New York, 1993; pp 205−221.
(89) Delescluse, C.; Cavey, M. T.; Martin, B.; Bernard, B. A.; Reichert, U.; Maignan, J.; Darmon, M.; Shroot, B. Selective High Affinity Retinoic Receptor α or β-γ Ligands. *Mol. Pharmacol.* **1991**, *40*, 556−562.
(90) Eagleyes-Ribot, T.; Chandraratna, R. A. S.; Lew-Kaya, D.; Sefton, J.; Duvic, M. J. Response of Psoriasis to a New Topical Retinoid, AGN190168. *J. Am. Acad. Dermatol.* **1994**, *30*, 581−590.
(91) Bernard, B. A.; Bernardon, J.-M.; Delescluse, C.; Martin, B.; Lenoir, M.-C.; Maignan, J.; Charpentier, B.; Pilgrim, W. R.; Reichert, U.; Shroot, B. Identification of Synthetic Retinoids with Selectivity for Human Nuclear Retinoic Acid Receptor γ. *Biochem. Biophys. Res. Commun.* **1992**, *186*, 977−983.
(92) Lehmann, J. M.; Jong, L.; Fanjul, A.; Cameron, J. F.; Lu, X. P.; Haefner, P.; Dawson, M. I.; Pfahl, M. Retinoids Selective for Retinoid X Receptor Response Pathways. *Science* **1992**, *258*, 1944.
(93) Boehm, M. F.; Zhang, L.; Badea, B. A.; White, S. K.; Mais, D. E.; Berger, E.; Suto, C. M.; Goldman, M. E.; Heyman, R. A. Synthesis and Structure Activity Relationships of Novel Retinoid X Receptor Selective Retinoids. *J. Med. Chem.* **1994**, *37*, 2930−2941.
(94) Apfel, C.; Bauer, F.; Crettaz, M.; Forni, L.; Kamber, M.; Kaufmann, F.; LeMotte, P.; Pirson, W.; Klaus, M. A. Retinoic Acid Receptor α Antagonist Selectivity Counteracts Retinoic Acid Effects. *Proc. Natl. Acad. Sci. U.S.A.* **1992**, *89*, 7129−7133.
(95) Kaneko, S.; Kagechika, H.; Kawachi, E.; Hashimoto, Y.; Shudo, K. Retinoid Antagonists. *Med. Chem. Res.* **1991**, *1*, 220−225.
(96) Eyrolles, L.; Kawachi, E.; Matsushima, Y.; Nakajima, O.; Kagechika, H.; Hashimoto, Y.; Shudo, K. Retinoid Antagonists: Molecular Design Based on the Ligand Superfamily Concept. *Med. Chem. Res.* **1992**, *2*, 361−367.
(97) Angulo, A.; Suto, C.; Boehm, M. F.; Heyman, R. A.; Ghazal, P. Retinoid Activation of Retinoic Acid Receptors but Not of Retinoid X Receptors Promotes Cellular Differentiation and Replication of Human Cytomegalovirus in Embryonal Cells. *J. Virol.* **1995**, *69*, 3831−3837.
(98) Gross, J.; Pitt-Rivers, R. Triiodothyronine in Relation to Thyroid Physiology. *Recent Prog. Horm. Res.* **1954**, *10*, 109−128.
(99) Hodin, R. A.; Lazar, M. A.; Wintman, B. I.; Darling, D. S.; Koeing, R. J.; Larsen, P. R.; Moore, D. D.; Chin, W. W. Identification of a Thyroid Hormone Receptor that is Pituitary-Specific. *Science* **1989**, *244*, 76−79.
(100) Yen, P. M.; Sunday, M. E.; Darling, D. S.; Chin, W. W. Isoform-Specific Thyroid Hormone Receptor Antibodies Detect Multiple Thyroid Hormone Receptors in Rat and Human Pituitaries. *Endocrinology* **1992**, *130*, 1539−1546.
(101) Chang, C. S.; Kokontis, J.; Acakpo-Satchivi, L.; Liao, S. S.; Takeda, H.; Chang, Y. Molecular Cloning of a New Human TR2 Receptor: a Class of Steroid Receptors with Multiple Ligand-Binding Domains. *Biochem. Biophys. Res. Commun.* **1989**, *165*, 735−741.
(102) Ritchie, H. H.; Wang, L.-H.; Tsai, S.; O'Malley, B. W.; Tsai, M.-J. COUP-TF Gene: a Structure Unique for the Steroid/Thyroid Receptor Superfamily. *Nucl. Acid Res.* **1990**, *18*, 6857−6862.
(103) Ladias, J. A. A.; Karathanasis, S. K. Regulation of the Apolipoprotein AI Gene by ARP-1, a Novel Member of the Steroid Receptor Family. *Science* **1991**, *251*, 561−565.
(104) Sladek, F. M.; Zhong, W.; Lai, E.; Darnell, J. E., Jr. Liver-Enriched Transcription Factor HNF-4 is a Novel Member of the Steroid Hormone Receptor Superfamily. *Genes Dev.* **1990**, *4*, 2353−2365.
(105) Issemann, I.; Green, S. Activation of a Member of the Steroid Hormone Receptor Superfamily by Peroxisome Proliferators. *Nature* **1990**, *347*, 645−650.
(106) Milbrandt, J. Nerve Growth Factor Induces a Gene Homologous to the Glucocorticoid Receptor Gene. *Neuron* **1988**, *1*, 183−188.
(107) Hazel, T. G.; Nathan, D.; Lau, L. F. A Gene Inducible by Serum Growth Factors Encodes a Member of the Steroid and Thyroid Hormone Receptor Superfamily. *Proc. Natl. Acad. Sci. U.S.A.* **1988**, *85*, 8444−8448.
(108) Giguere, V.; Tini, M.; Flock, G.; Ong, E.; Evans, R.; Otulakowski, G. Isoform-Specific Amino-Terminal Domains Dictate DNA Binding Properties of ROR, a Novel Family of Orphan Hormone Nuclear Receptors. *Genes Dev.* **1994**, *8*, 538−553.
(109) Carlberg, C.; Hooft van Huijsduijnen, R.; Staple, J. K.; DeLamarter, J. F.; Becker-Andre, M. RZRs, a New Family of Retinoid-Related Orphan Receptors that Function as both Monomers and Homodimers. *Mol. Endocrinol.* **1994**, *8*, 757−770.
(110) Denner, L. A.; Weigel, N. L.; Maxwell, B. L.; Schrader, W. T.; O'Malley, B. W. Regulation of Progesterone Receptor-Mediated Transcription by Phosphorylation. *Science* **1990**, *250*, 1740−1743.
(111) Darnell, J. E., Jr.; Kerr, I. M.; Stark, G. R. JAK-STAT Pathways and Transcriptional Activation in Response IFNs and Other Extracellular Signaling Proteins. *Science* **1994**, *264*, 1415−1421.
(112) Levy, D.; Darnell, J. E., Jr. Interferon-Dependent Transcriptional Activation: Signal Transduction without Second Messenger Involvement? *New Biol.* **1990**, *2*, 923−928.
(113) Ihle, J. N.; Witthuhn, B. A.; Quelle, F. W.; Yamamoto, K.; Thierfelder, W. E.; Kreider, B.; Silvennoinen, O. Signaling by the Cytokine Receptor Superfamily: JAKs and STATs. *Trends Biochem. Sci.* **1994**, *19*, 222−227.
(114) Wilks, A. Two Putative Protein-Tyrosine Kinases Identified by Application of The Polymerase Chain Reaction. *Proc. Natl. Acad. Sci. U.S.A.* **1989**, *86*, 1603−1607.
(115) Firmbach-Kraft, I. Tyk2, Prototype Of A Novel Class of Non-Receptor Tyrosine Kinase Genes. *Oncogene* **1990**, *5*, 1329−1336.
(116) Koch, C. A.; Anderson, D.; Moran, M. F.; Ellis, C.; Pawson, T. SH2 and SH3 Domains: Elements that Control Interactions of Cytoplasmic Signaling Proteins. *Science* **1991**, *252*, 668−674.
(117) Lamb, P.; Haslam, J.; Kessler, L.; Seidel, H. M.; Stein, R. B.; Rosen, J. Rapid Activation of the Interferon-γ Signal Transduction Pathway by Inhibitors of Tyrosine Phosphatases. *J. Interferon Res.* **1994**, *14*, 365−373.
(118) Greenlund, A. C.; Farrar, M. A.; Viviano, B. L.; Schreiber, R. D. Ligand-induced IFNγ Receptor Tyrosine Phosphorylation Couples the Receptor to its Signal Transduction System (p91). *EMBO J.* **1994**, *13*, 1591−1600.

(119) Schindler, C.; Fu, X.-Y.; Improta, T.; Abersold, R.; Darnell, J. E., Jr. Proteins of Transcription Factor ISGF-3, the Interferon α-Induced Transcriptional Activator. *Proc. Natl. Acad. Sci. U.S.A.* **1992**, *89*, 7836–40.
(120) Seidel, H. M.; Milocco, L. H.; Lamb, P.; Darnell, J. E., Jr.; Stein, R. B.; Rosen, J. Spacing of Palindromic Half Sites as a Determinant of Selective DNA Binding and Transcriptional Activity of Signal Transducers and Activators of Transcription. *Proc. Natl. Acad. Sci. U.S.A.* **1995**, *92*, 3041–3045.
(121) Levitzki, A.; Cazit, A. Tyrosine Kinase Inhibition: An Approach to Drug Development. *Science* **1995**, *267*, 1782–1788.
(122) Fry, D. W.; Kraker, A. J.; McMichael, A.; Ambroso, L. A.; Nelson, J. M.; Leopold, W. R.; Connors, R. W.; Bridges, A. J. A Specific Inhibitor of Epidermal Growth Factor Receptor Tyrosine Kinase. *Science* **1994**, *265*, 1093–1096.
(123) Beebe, J. S.; Darling, D. S.; Chin, W. W. 3,5,3′-Triiodothyronine Receptor Auxiliary Protein (TRAP) Enhances Receptor Binding by Interactions Within the Thyroid Hormone Response Element. *Mol. Endocrinol.* **1991**, *5*, 85–93.
(124) Kupfer, S. R.; Marschke, K. B.; Wilson, E. M.; French, F. S. Receptor Accessory Factor Enhances Specific DNA Binding of Androgen and Glucocorticoid Receptors. *J. Biol. Chem.* **1993**, *268*, 17519–27.

JM950212K

Label and Approval History

Drug Name(s) TESTODERM (Brand Name Drug)

FDA Application No. (NDA) 019762

Active Ingredient(s) TESTOSTERONE

Company ALZA

Go to Approval History

Label Information

What information does a label include?

Note: Not all labels are available in electronic format from FDA.

Labels are not available on this site for TESTODERM, NDA no. 019762

Approval History

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Older labels are for historical information only, and should not be used for clinical purposes.
Action dates can only be verified from 1984 to the present.

Action Date	Supplement Number	Approval Type	Letters, Reviews, Labels, Patient Package Insert	Note
12/17/1997	005	Control Supplement		This supplement type does not usually require new labeling.
05/12/1997	004	Control Supplement		This supplement type does not usually require new labeling.
06/26/1996	001	Formulation Revision		Label is not available on this site.
05/28/1996	002	Labeling Revision		Label is not available on this site.
10/12/1993	000	Approval		Label is not available on this site.

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Label and Approval History

Drug Name(s) ANDRODERM (Brand Name Drug)

FDA Application No. (NDA) 020489

Active Ingredient(s) TESTOSTERONE

Company WATSON LABS

Go to Approval History

Label Information

What information does a label include?

Note: Not all labels are available in electronic format from FDA.

Labels are not available on this site for ANDRODERM, NDA no. 020489

Approval History

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Action dates can only be verified from 1984 to the present.

Action Date	Supplement Number	Approval Type	Letters, Reviews, Labels, Patient Package Insert	Note
11/27/2002	011	Formulation Revision		Label is not available on this site.
08/09/2002	010	Labeling Revision	Letter	Label is not available on this site.
01/15/2002	007	Labeling Revision	Letter	Label is not available on this site.
10/24/2000	009	Manufacturing Change or Addition		This supplement type does not usually require new labeling.
07/13/2000	008	Control Supplement		This supplement type does not usually require new labeling.
10/09/1998	006	Formulation Revision		Label is not available on this site.
06/29/1998	005	Control Supplement		This supplement type does not usually require new labeling.
03/18/1998	004	Labeling Revision		Label is not available on this site.
05/02/1997	003	Control Supplement	Review	This supplement type does not usually require new labeling.
04/22/1997	002	Package Change		Label is not available

				on this site.
07/10/1996	001	Labeling Revision		Label is not available on this site.
09/29/1995	000	Approval		Label is not available on this site.

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Label and Approval History

Drug Name(s)	ANADROL-50 (Brand Name Drug)
FDA Application No.	(NDA) 016848
Active Ingredient(s)	OXYMETHOLONE
Company	UNIMED PHARMS

Go to Approval History

Label Information

What information does a label include?

Note: Not all labels are available in electronic format from FDA.

View the label approved on 08/25/2004 for ANADROL-50, NDA no. 016848

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Approval History

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Action dates can only be verified from 1984 to the present.

Action Date	Supplement Number	Approval Type	Letters, Reviews, Labels, Patient Package Insert	Note
08/25/2004	021	Labeling Revision	Label Letter	
11/17/2003	020	Labeling Revision	Letter	Label is not available on this site.
02/06/2002	019	Manufacturing Change or Addition		This supplement type does not usually require new labeling.
05/14/1997	018	Control Supplement		This supplement type does not usually require new labeling.
06/09/1994	017	Control Supplement		This supplement type does not usually require new labeling.
07/15/1993	016	Labeling Revision		Label is not available on this site.
07/15/1993	013	Labeling Revision		Label is not available on this site.

08/24/1992	014	Control Supplement		This supplement type does not usually require new labeling.
02/18/1992	015	Labeling Revision		Label is not available on this site.
08/14/1991	012	Labeling Revision		Label is not available on this site.
01/26/1990	011	Control Supplement		This supplement type does not usually require new labeling.
08/25/1986	010	Labeling Revision		Label is not available on this site.
01/18/1983	009	Control Supplement		This supplement type does not usually require new labeling.
04/15/1980	006	Labeling Revision		Label is not available on this site.
06/15/1976	005	Package Change		Label is not available on this site.
10/10/1975	004	Control Supplement		This supplement type does not usually require new labeling.
01/18/1972	000	Approval		Label is not available on this site.

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Label and Approval History

Drug Name(s) TESTRED (Generic Drug)

FDA Application No. (ANDA) 083976

Active Ingredient(s) METHYLTESTOSTERONE

Company VALEANT PHARM INTL

Go to Approval History

Label Information

What information does a label include?

Note: Not all labels are available in electronic format from FDA.

Labels are not available on this site for TESTRED, ANDA no. 083976

Approval History

Note: Not all reviews are available in electronic format from FDA.
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Action dates can only be verified from 1984 to the present.

Action Date	Supplement Number	Approval Type	Letters, Reviews, Labels, Patient Package Insert	Note
11/27/2002	027	Labeling		Label is not available on this site.
08/18/1998	026	Chemistry		Label is not available on this site.
08/25/1997	025	Chemistry		Label is not available on this site.
08/25/1997	024	Chemistry		Label is not available on this site.
08/08/1994	023	Labeling in Effect		Label is not available on this site.
11/03/1992	022	Labeling in Effect		Label is not available on this site.
05/29/1992	021	Labeling		Label is not available on this site.
05/25/1989	020	Labeling		Label is not available on this site.
03/08/1983	018	Chemistry		Label is not available on this site.
12/03/1973	000	Approval		Label is not available on this site.

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Label and Approval History

Drug Name(s) EULEXIN (Brand Name Drug)

FDA Application No. (NDA) 018554

Active Ingredient(s) FLUTAMIDE

Company SCHERING

Go to Approval History

Label Information

What information does a label include?

Note: Not all labels are available in electronic format from FDA.

View the label approved on 07/23/2001 for EULEXIN, NDA no. 018554

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Approval History

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Action Date	Supplement Number	Approval Type	Letters, Reviews, Labels, Patient Package Insert	Note
07/23/2001	023	Labeling Revision	Label	
03/30/2001	022	Labeling Revision	Letter Review	Label is not available on this site.
03/23/2001	021	Labeling Revision	Letter Review	Label is not available on this site.
03/14/2001	020	Control Supplement		This supplement type does not usually require new labeling.
01/23/2001	019	Manufacturing Change or Addition		This supplement type does not usually require new labeling.
01/07/2000	018	Manufacturing Change or Addition		This supplement type does not usually require new labeling.
08/19/1999	015	Labeling Revision		Label is not available on this site.

05/18/1998	017	Control Supplement		This supplement type does not usually require new labeling.
11/12/1997	016	Control Supplement		This supplement type does not usually require new labeling.
08/30/1996	004	Control Supplement		This supplement type does not usually require new labeling.
06/21/1996	014	New or Modified Indication		Label is not available on this site.
11/15/1995	013	Manufacturing Change or Addition		This supplement type does not usually require new labeling.
09/15/1995	012	Labeling Revision		Label is not available on this site.
10/26/1994	011	Labeling Revision		Label is not available on this site.
07/22/1993	008	Manufacturing Change or Addition		This supplement type does not usually require new labeling.
07/29/1992	002	Manufacturing Change or Addition		This supplement type does not usually require new labeling.
05/18/1992	009	Manufacturing Change or Addition		This supplement type does not usually require new labeling.
09/19/1991	010	Labeling Revision		Label is not available on this site.
05/07/1991	005	Control Supplement		This supplement type does not usually require new labeling.
09/03/1990	003	Labeling Revision		Label is not available on this site.
10/17/1989	001	Labeling Revision		Label is not available on this site.
01/27/1989	000	Approval		Label is not available on this site.

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Label and Approval History

Drug Name(s) CASODEX (Brand Name Drug)

FDA Application No. (NDA) 020498

Active Ingredient(s) BICALUTAMIDE

Company ASTRAZENECA

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Label Information

What information does a label include?

Note: Not all labels are available in electronic format from FDA.

View the label approved on 05/11/2005 for CASODEX, NDA no. 020498

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Action Date	Supplement Number	Approval Type	Letters, Reviews, Labels, Patient Package Insert	Note
05/11/2005	016	Labeling Revision	Label Letter	
09/23/2002	013	Manufacturing Change or Addition		This supplement type does not usually require new labeling.
01/25/2002	011	Control Supplement		This supplement type does not usually require new labeling.
10/25/2001	010	Manufacturing Change or Addition		This supplement type does not usually require new labeling.
02/28/2001	008	Labeling Revision		Label is not available on this site.
12/11/2000	007	Manufacturing Change or Addition		This supplement type does not usually require new labeling.
11/04/1999	005	Manufacturing Change or Addition		This supplement type does not usually require new labeling.

12/12/1997	004	New or Modified Indication		Label is not available on this site.
11/12/1997	003	Control Supplement		This supplement type does not usually require new labeling.
10/04/1995	000	Approval		Label is not available on this site.

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STUDY

Topical Cyproterone Acetate Treatment in Women With Acne

A Placebo-Controlled Trial

Doris M. Gruber, MD; Michael O. Sator, MD; Elmar A. Joura, MD; Eva Maria Kokoschka, MD; Georg Heinze, MSc; Johannes C. Huber, MD, PhD

Objective: To evaluate the clinical and hormonal response of topically applied cyproterone acetate, oral cyproterone acetate, and placebo lotion in women with acne.

Design: Placebo-controlled, randomized study.

Setting: Patients were recruited from the Institute of Endocrine Cosmetics, Vienna, Austria.

Patients: Forty women with acne.

Interventions: Treatment with oral medication consisting of 0.035 mg of ethinyl estradiol and 2 mg of cyproterone acetate (n=12), 20 mg of topical cyproterone acetate lotion (n=12), and placebo lotion (n=16) was offered. Patients were assessed monthly for 3 months.

Main Outcome Measures: Clinical grading according to acne severity and lesion counts as well as determinations of serum cyproterone acetate concentrations.

Results: After 3 months of therapy with topical cyproterone acetate, the decrease of mean facial acne grade from 1.57 to 0.67 was significantly better ($P<.05$) compared with placebo (which showed a change from 1.57 to 1.25), but not compared with oral medication (1.56 to 0.75) ($P>.05$). Lesion counts also decreased from 35.9 to 9.1 in the topical cyproterone acetate group compared with oral medication (45.4 to 15.5) ($P>.05$) and placebo (38.2 to 23.1) ($P<.05$). After topical cyproterone acetate treatment, serum cyproterone acetate concentrations were 10 times lower than those found after oral cyproterone acetate intake.

Conclusions: The therapeutic effect of topically applied cyproterone acetate for acne treatment was clearly demonstrated. Topically applied sexual steroids in combination with liposomes are as effective as oral antiandrogen medication in acne treatment, while reducing the risk of adverse effects and avoiding high serum cyproterone acetate concentrations.

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HORMONAL approaches to the treatment of acne have been the subject of much interest ever since the sebaceous gland was demonstrated to be sensitive to androgens. The use of topical antiandrogens has had theoretical appeal since the pilosebaceous unit was found to be an androgen-dependent structure. Unfortunately, however, this theory has not been translated into clinical practice.

17β-Estradiol and progesterone have long been applied topically in the treatment of acne. The reasons for using topical rather than oral therapy include the avoidance of hepatic metabolism, the reduced risk of systemic adverse effects, the ability to combat acne in both female and male patients, and the lack of a contraceptive effect in women. It is known that cyproterone acetate is effective when given orally. However, trials using topical cyproterone acetate have not proved successful because of the lack of a suitable vehicle for transdermal treatment.[1,2] Cyproterone acetate is a potent steroidal antiandrogen with progestational activity. It is used alone or in combination with ethinyl estradiol or estradiol valerate in the treatment of women suffering from disorders associated with androgenization, eg, acne or hirsutism. Cyproterone acetate competes with dihydrotestosterone for the androgen receptor and inhibits translocation of the hormone receptor complex into the cell nucleus.[3]

This study was undertaken to consider once again a cyproterone acetate formulation delivered to the skin surface, thus approaching the target cell topically and bypassing the enterohepatic circulation. The aim of this open, placebo-controlled study was to compare the effectiveness of topical cyproterone acetate, an oral formulation containing 0.035 mg of ethinyl estradiol and 2 mg of cyproterone acetate (Dianemite, Schering, Vienna, Austria), and a placebo preparation in women with mod-

PATIENTS AND METHODS

Forty-five women aged between 26 and 38 years (mean age, 30.3 years) with moderate to severe acne who consulted the endocrinology outpatient department for a hormonal evaluation and treatment of their acne were enrolled in this 3-month trial.

Informed consent was obtained from all the patients, and the study was approved by the local ethics committee. Patients with medical contraindications to the therapy or unwilling to smoke less than 5 cigarettes daily were not included in the study. Patients were required to use barrier contraception during the treatment period. All acne medication had been stopped 6 weeks before the commencement of the study.

During the initial visit, the patients were randomly assigned to 1 of the 3 treatment groups. One group (n=12) received an oral contraceptive regimen for 3 months, with administration of a daily dose of 0.035 mg of ethinyl estradiol and 2 mg of cyproterone acetate for the first 21 days of the menstrual cycle, followed by a 7-day pill-free interval. The first treatment cycle started on the first day of menstrual bleeding. The second group (n=12) used a topical cyproterone acetate lotion, with treatment starting on the first day of menstrual bleeding and being taken continuously for 3 months. The topical lotion was self-applied once daily in the evening, to the face only, with a calibrated pipette. The daily amount of cyproterone acetate liposome lotion used was 10 mL, containing 20 mg of cyproterone acetate. Liposome lotion consisted of soybean oil, eilecithin, glycerol, and oleic acid (Leopold Pharma, Graz, Austria).

The third group (n=16) used a placebo liposome lotion containing soybean oil, eilecithin, glycerol, and oleic acid without the active substance; it was applied as in the topical cyproterone acetate group.

Acne severity on the face was graded according to the method of Burke and Cunliffe,[4] with the individual scores added up to yield a total result. Four sites on the face (chin, forehead, and left and right cheeks) were graded. The mean value from this grading was used for further calculations. In addition, numbers of macules, papules, pustules, nodules, and cysts on the face were counted by the same dermatologist (E.M.K.) at each visit. Lesion counts were defined as the total of comedones and inflammatory lesions. Only 3 of the patients had few inflammatory lesions, and their counts were thus added to the noninflammatory lesions. Acne on other parts of the body was not evaluated in this study. Assessments were done during the midpoint of the menstrual cycle, ie, between days 10 and 19, with day 1 being defined as the first day of menstrual bleeding. Before commencement of the trial, all patients underwent 2 baseline examinations performed 1 week apart. Progress assessments were made midway through the first, second, and third cycles of treatment. Forty women completed the trial, and their data were included in the final analysis. Five patients dropped out before the end of treatment.

TOPICAL APPLICATION OF CYPROTERONE ACETATE

The application system was similar to that used for topical 17β-estradiol and topical 17α-dihydrotestosterone studies. Pharmacokinetic details for these steroidal substances have been described previously.[5,6] Because cyproterone acetate is a lipophilic steroid, a similar absorption rate was assumed. The daily transdermal cyproterone acetate dosage was 20 mg, based on a similar formulation combining soybean oil, eilecithin, glycerol, and oleic acid.

Liposomes were used to improve the transdermal penetration of the antiandrogen. Topical application of cyproterone acetate in patients with acne had been attempted before, but the clinical results were discouraging.

SERUM CYPROTERONE ACETATE LEVELS

Serum levels of cyproterone acetate were determined in 5 patients in the topical cyproterone acetate group both before treatment and 3 months after study entry, with blood samples taken exactly 45 minutes after the last topical cyproterone acetate application. Baseline serum cyproterone acetate concentrations were below the detection limit. Serum cyproterone acetate levels were measured by radioimmunoassay, by a dextran-coated charcoal method. Aliquots of serum (0.1 mL) were diluted with physiological saline to a final volume of 0.5 mL. After extraction with 2.5 mL of diethyl ether, the ether phase was separated and fully evaporated, and the residue was reconstituted with 0.8 mL of an assay buffer. This solution was incubated with 0.1 mL of tritiated cyproterone acetate (specific activity, 0.84 GBq/mg; Schering AG, Berlin, Germany) and 0.1 mL of antiserum (batch C003, Schering AG) at 4°C for 16 hours, mixed with 0.2 mL of dextran-coated charcoal, and vortexed. After 15 minutes at 4°C, the mixture was centrifuged. The supernatants were decanted into liquid scintillation vials and mixed with 4.5 mL of scintillation cocktail. The radioactivity was measured on a scintillation counter. To obtain the standard curves, 1 mg of cyproterone acetate was dissolved in methanol and diluted with assay buffer, yielding final concentrations ranging from 39 to 10 000 pg/mL. In addition, a drug-free sample (0 pg/mL) was used. Calibration and samples were analyzed in duplicates. A spline function was used for the evaluation of the data. The interassay and intra-assay coefficients ranged from 5.0% to 13.0%. The mean blank value for blank control samples was found to be 28 pg/mL.

STATISTICAL METHODS

Overall significance of the group effect was evaluated by analysis of variance. Pairwise comparisons between groups were made by Student *t* tests for mean comparison. A *P* value less than .05 was considered to indicate significance. The program used for statistical analysis was SAS/PROC GLM (SAS/STAT software, version 6, SAS Institute Inc, Cary, NC, 1989).

erate to severe acne. Moreover, we evaluated in a preliminary study the serum cyproterone acetate levels after transdermal application and compared them with levels obtained by administration of an oral preparation containing 0.035 mg of ethinyl estradiol and 2 mg of cyproterone acetate.

RESULTS

Of the 45 patients who entered the study, 40 completed the 3 cycles of treatment and were included in the analysis (12 receiving oral cyproterone acetate, 12 receiving topical cyproterone acetate, and 16 receiving

Table 1. Clinical Features of Patients in the Trial

Therapy	No. of Patients Entering Trial (No. Completing)	Mean (Range)									
				Facial Acne Grade				Lesion Count			
		Age, y	Weight, kg	Baseline	1 mo	2 mo	3 mo	Baseline	1 mo	2 mo	3 mo
Oral cyproterone acetate	14 (12)	29.4 (26-37)	58.2 (55.7-61.4)	1.56 (1.55-1.58)	1.22 (1.18-1.24)	1.15 (1.12-1.17)	0.75 (0.20-0.97)	45.4 (30-58)	33.5 (21-42)	24.8 (16-35)	15.5 (11-21)
Topical cyproterone acetate	13 (12)	31.3 (26-38)	63.1 (55.3-77.3)	1.57 (1.56-1.58)	1.16 (1.10-1.18)	1.12 (1.10-1.15)	0.67 (0.59-0.80)	35.9 (28-59)	21.8 (12-40)	17.7 (9-33)	9.1 (3-20)
Placebo	18 (16)	30.3 (26-38)	67.0 (55.1-77.5)	1.57 (1.54-1.59)	1.39 (1.30-1.45)	1.30 (1.28-1.40)	1.25 (1.22-1.28)	38.2 (29-47)	32.4 (25-42)	27.6 (21-35)	23.1 (17-30)

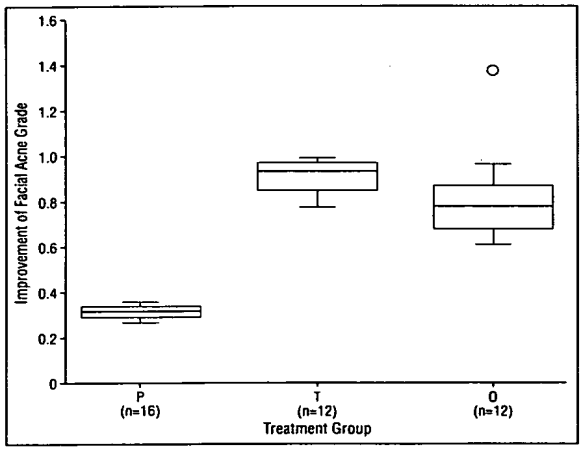

Figure 1. *Boxplot of response of the 3 treatment groups in facial acne grades during 3 months of treatment. Mean values (ranges) are as follows: group T, 0.90 (0.77-0.99); group O, 0.81 (0.61-1.37); and group P, 0.32 (0.27-0.36).*

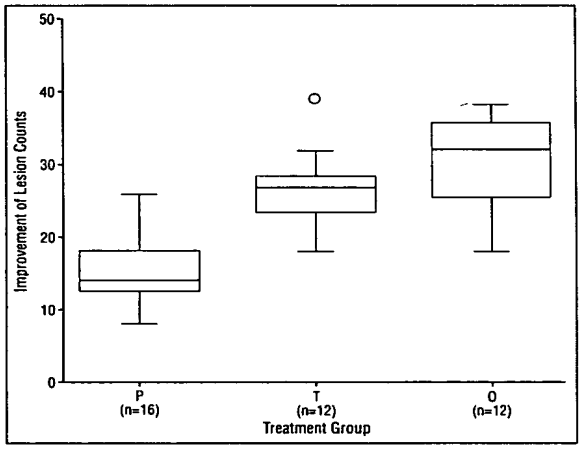

Figure 2. *Boxplot of response of the 3 treatment groups in lesion counts during 3 months of treatment. Means (ranges) are as follows: group T, 26.8 (18.0-39.0); group O, 29.9 (18.0-38.0); and group P, 15.1 (8.0-26.0).*

placebo). Two patients in the oral cyproterone acetate group dropped out of the study because they had failed to take the tablets regularly. One patient was excluded from the topical cyproterone acetate group because of a moderate local reaction and because she was not willing to use the mechanical contraception. Two patients in the placebo group withdrew from the study for personal reasons. The patients' ages, their initial mean acne gradings, and lesion counts are listed in **Table 1**.

Response to therapy was measured by calculating the difference between baseline measurements and measurements after 3 months of therapy. During the 3-month assessment period, both oral and topical cyproterone acetate were found to be significantly more effective than the placebo preparation in reducing mean facial acne grades and lesion counts ($P<.05$ for both groups). No significant differences in treatment success were observed between the oral and topical therapy ($P>.05$) (**Figure 1** and **Figure 2**).

After 3 months of topical cyproterone acetate application, lesion counts had decreased from a mean of 35.9 (range, 28-59) to 9.1 (3-20) ($P<.05$). The serum levels of cyproterone acetate are shown in **Table 2**. Serum cyproterone acetate concentrations were below the detection limit of 28 pg/mL before the treatment but increased to values of between 119 and 311 pg/mL after topical cyproterone acetate application. No subjective adverse effects or bleeding irregularities were observed in either of the groups during the 3 months of treatment.

Table 2. Serum Cyproterone Acetate Levels Before and After Topical Cyproterone Acetate Therapy in 5 Patients

Patient No./ Age, y	Serum Cyproterone Acetate, pg/mL	
	Before Treatment	After 3 mo of Treatment
1/34	<28*	226
2/37	<28	281
3/26	<28	286
4/35	<28	311
5/30	<28	119

**Detection limit.*

COMMENT

Acne is a common disorder in young females and males, and the therapeutic strategies available at the moment are not always sufficient and without shortcomings. Therefore, there is still a need for more effective topical therapies, particularly those that are free of harmful ad-

verse effects and easy to handle. There are numerous well-established hormonal and nonhormonal approaches to the treatment of acne. As acne is known to be an androgen-dependent disorder, oral antiandrogen treatment has been shown to be successful. In the present study, we assessed the efficiency of topical cyproterone acetate in patients afflicted with acne. Although the number of subjects in this study was small, patients were specifically selected from a large group of patients with acne on the basis of having moderate to severe lesion scores, and no other visual evidence of hyperandrogenism. Moreover, none of the patients had received any systemic treatment for 6 weeks before the commencement of the study.

THE USE OF topical antiandrogens in the treatment of acne has been intensively investigated ever since the pilosebaceous unit was found to be androgen dependent. In 1969, Cunliffe et al[1] used topical cyproterone acetate dispensed in dimethyl sulfoxide in 12 patients but found no improvement because of the lack of a suitable vehicle for the steroids. Seven years later, Pye et al[2] used 1% cyproterone acetate suspended in cetomacrogol cream BPC (formula A), but none of the patients showed any marked acne improvement. Meanwhile, a wealth of experience in the area of acne treatment has been gained by various clinicians and researchers. There are several alternatives to cyproterone acetate. Estrogens, progesterone, spironolactone, flutamide, and gonadotropin-releasing hormone analogues have all been used in various concentrations in clinical trials.[7-9] These substances reduce sebaceous gland activity leading to a clinical improvement of acne. However, they have 1 problem in common. They are ingested and thus affect the entire human organism, producing systemic adverse effects.

It is well known that oral estrogens effectively suppress sebum excretion and improve acne in both men and women. However, the doses required to produce a therapeutic effect are associated with unacceptable adverse effects.[10] The effect of topical progesterone also has been evaluated; whereas a reduction in the sebum excretion rate was demonstrated in female patients, no change was seen in male patients. Moreover, the sebosuppressive effect was lost after 3 months.[11]

Antiandrogens show the most likely source of therapeutic success in the hormonal manipulation of the sebaceous gland.[12] Cyproterone acetate is most often prescribed in combination with ethinyl estradiol, with the maximum clinical effect generally seen between the third and sixth months of treatment. Oral spironolactone also has been shown both to decrease sebum excretion rates and to improve clinical acne.[13] However, the prescription of spironolactone in this indication has been markedly reduced since the publication of animal research data indicating that it may cause breast cancer in rats.[14] The oral nonsteroidal antiandrogen flutamide has been found to reduce acne but it is not suitable for clinical use.[15] Chlormadinone acetate is a 19-norprogestin with antiandrogenic properties. It is used in combination with ethinyl estradiol as an oral contraceptive and has proved to be successful in cases of mild acne and seborrhea.[16] Dienogest, apparently the first 19-nortestosterone derivative with antiandrogenic effects, is currently used in oral contraceptives and has been suggested for antiandrogen treatment. However, clinical trials evaluating the antiandrogen property of this substance are inadequate.[17]

Finally, the new topical nonsteroidal antiandrogen inocoterone acetate has produced only modest clinical effects in the treatment of acne.[18] Evidence suggests that not all patients with acne exhibit elevated serum androgen levels.[19] Rather, several studies have indicated an increased local formation of androgens, disturbances of the androgen metabolism,[20] or an increased sensitivity of the androgen receptor to normal levels of androgens.[21] Consequently, antiandrogens and 5α-reductase inhibitors may also play a role in the treatment of clinically hyperandrogenemic women.[22]

Despite the lack of success with topical cyproterone acetate in previous studies, we undertook a placebo-controlled trial using a liposome lotion as a carrier for cyproterone acetate to overcome the difficulty of delivering the active substance to the target cell. In the present study, the therapeutic effectiveness of topically applied cyproterone acetate in cases of acne was clearly demonstrated. Mean facial acne grades and lesion counts decreased significantly with both topical and oral cyproterone acetate. The preliminary serum determinations confirmed our initial expectation that topical use would result in lower serum cyproterone acetate levels than would oral cyproterone acetate intake. In fact, cyproterone acetate levels were 10 times lower after topical than after oral application, while producing a similar clinical response. According to data in the literature, cyproterone acetate levels after oral intake of 2 mg of cyproterone acetate can be expected to be an order of magnitude higher (4073 pg/mL); however, this is because a contraceptive effect is required.[23] Further studies and pharmacokinetic investigations are necessary to determine the optimal dosage and carrier regimens.

The mechanisms responsible for the effectiveness of topical cyproterone acetate are outside the scope of the present study. Whether topical cyproterone acetate acts directly on the skin or whether the serum levels, although low, are responsible for its therapeutic effects will have to be determined in further studies.

Notably, a significant therapeutic improvement compared with the placebo group was seen after only 3 months of treatment with both oral and topical cyproterone acetate. In summary, the results of this study suggest that topical application of the antiandrogen cyproterone acetate in the treatment of acne is as effective as oral cyproterone acetate in combination with ethinyl estradiol, provided that a suitable carrier is used. The liposome lotion we used unlocks the potential of topical cyproterone acetate application and releases the benefits of reduced adverse effects, at least within the treatment period evaluated in this study. Thus, the cyproterone acetate lotion represents a suitable alternative or additional local treatment for women with acne.

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REFERENCES

1. Cunliffe WJ, Shuster S, Cassels Smith AJ. The effect of topical cyproterone acetate on sebum secretion in patients with acne. *Br J Dermatol.* 1969;81:200-201.
2. Pye RJ, Burton JL, Harris JI. Effect of 1% cyproterone acetate in cetomacrogol cream BPC (formula A) on sebum excretion rate in patients with acne. *Br J Dermatol.* 1976;95:427-428.
3. Sciarra F, Toscano V, Concolino G, Di Silverio F. Androgens: clinical applications. *J Steroid Biochem Mol Biol.* 1990;37:349-362.
4. Burke BM, Cunliffe WJ. The assessment of acne vulgaris: the Leeds technique. *Br J Dermatol.* 1984;111:83-87.
5. Fiet J, Morville R, Chemama D, et al. Percutaneous absorption of 5 alpha dihydrotestosterone in man, I: plasma androgen and gonadotropin levels in normal adult men after percutaneous administration of 5 alpha dihydrotestosterone. *Int J Androl.* 1982;5:586-594.
6. Scott RT, Ross B, Anderson S, et al. Pharmacokinetics of percutaneous estradiol: a crossover study using a gel and a transdermal system in comparison with oral micronized estradiol. *Obstet Gynecol.* 1991;77:758-764.
7. Muhlemann MF, Carter GD, Cream JJ, et al. Oral spironolactone: an effective treatment of acne vulgaris in women. *Br J Dermatol.* 1986;115:227-232.
8. Adashi EY. Potential utility of gonadotropin-releasing hormone agonist in the management of ovarian hyperandrogenism. *Fertil Steril.* 1990;53:765-779.
9. Cusan I, Dupont A, Gomez LL, Tremblay RR, Labrie F. Comparison of flutamide and spironolactone in the treatment of hirsutism: a randomized controlled trial. *Fertil Steril.* 1994;61:281-287.
10. Strauss JS, Pochi PE. The effect of cyclic progestogen-estrogen therapy on sebum and acne in women. *JAMA.* 1964;190:815-819.
11. Simpson NB, Bowden PE, Forster RA, et al. The effect of topically applied progesterone on sebum excretion rate. *Br J Dermatol.* 1979;100:687-692.
12. Dorfmann RI. Biological activity of antiandrogens. *Br J Dermatol.* 1970;82 (suppl 6):3-8.
13. Burke BM, Cunliffe WJ. Oral spironolactone therapy for female patients with acne, hirsutism or androgenetic alopecia. *Br J Dermatol.* 1985;112:124-125.
14. Asscher AW. Spironolactone. *Curr Probl.* 1988;21:3-4.
15. Cusan L, Dupont A, Belanger A, Tremblay R, et al. Treatment of hirsutism with the pure antiandrogen flutamide. *J Am Acad Dermatol.* 1990;23:462-469.
16. Nürnberger F. Comparison of Diane-35 with Neo-Eunomin from the dermatological point of view. In: Schindler AE, ed. *Antiandrogen-Östrogentherapie bei Androgenisierungserscheinungen.* Berlin, Germany: Walter de Gruyter; 1988:205-214.
17. Grunwald K, Rabe T, Feldmann K, Runebaum B. Behandlung von Androgenisierungserscheinungen der Frau: eine Übersicht. *Fertilität.* 1995;11:82-89.
18. Lookingbill DP, Abrams BB, Ellis CN, et al. The effect of a topical antiandrogen inocoterone acetate (RU 882) on acne: results of a multicenter clinical trial. *Arch Dermatol.* 1992;128:1197-1200.
19. Carmina E, Lobo RA. Evidence for increased androsterone metabolism in some normoandrogenemic women with acne. *J Clin Endocrinol Metab.* 1993;76:1111-1114.
20. Joura EA, Geusau A, Schneider B, Söregi G, Huber JC. Serum 3 α-androstanediol-glucuronide is decreased in nonhirsute women with acne vulgaris. *Fertil Steril.* 1996;66:1033-1035.
21. Labrie F. Intracrinology. *Mol Cell Endocrinol.* 1991;78:229-233.
22. Ciotta L, Cianci A, Calogero AE, et al. Clinical and endocrine effects of finasteride, a 5α-reductase inhibitor, in women with idiopathic hirsutism. *Fertil Steril.* 1995;64:299-306.
23. Kuhnz W, Staks T, Jütting G. Pharmacokinetics of cyproterone acetate and ethinylestradiol. *Contraception.* 1993;48:557-575.

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A Prospective Randomized Trial Comparing Low Dose Flutamide, Finasteride, Ketoconazole, and Cyproterone Acetate-Estrogen Regimens in the Treatment of Hirsutism

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ABSTRACT

Sixty-six hirsute women were randomized and treated with 1) flutamide (n = 15), 250 mg/day; 2) finasteride (n = 15), 5 mg/day; 3) ketoconazole (n = 16), 300 mg/day; and 4) ethinyl estradiol (EE)-cyproterone acetate (CPA; n = 20), 0.01 mg EE/day for the first week, 0.02 mg EE/day for the second week, and 0.01 mg EE/day for the third week, followed by a pause of 7 days, then 12.5 mg CPA/day added during the first 10 days of every month for 12 months. Hirsutism was evaluated by the Ferriman-Gallwey score, and hair diameter and hair growth rate were determined by a special image analysis processor in basal conditions and after 90, 180, 270, and 360 days of treatment. All treatments produced a significant decrease in the hirsutism score, hair diameter, and daily hair growth rate: flutamide, $-55 \pm 13\%$, $-21 \pm 14\%$, and $-37 \pm 18\%$; finasteride, $-44 \pm 13\%$, $-16 \pm 12\%$, and $-27 \pm 14\%$; ketoconazole, $-53 \pm 18\%$, $-14 \pm 12\%$, and $-30 \pm 21\%$; and EE-CPA, $-60 \pm 18\%$, $-20 \pm 11\%$, and $-28 \pm 21\%$.

Some differences existed among treatments with regard to effectiveness; EE-CPA and flutamide seem to be the most efficacious in improving hirsutism. For the hirsutism score, a greater decrease was seen with EE-CPA ($-60 \pm 18\%$) than with finasteride ($-44 \pm 13\%$; $P < 0.01$) and a greater decrease was seen with flutamide ($-58 \pm 18\%$) than with finasteride ($-44 \pm 13\%$; $P < 0.05$). Flutamide is the fastest in decreasing hair diameter; EE-CPA is the fastest in slowing down hair growth, even though at the end of the treatment there was a significant difference between flutamide and finasteride only ($-41 \pm 18\%$ *vs.* $-27 \pm 14\%$; $P < 0.05$).

Flutamide, ketoconazole, and EE-CPA induced a significant decrease in total and free testosterone, 5α-dihydrotestosterone, dehydroepiandrosterone, dehydroepiandrosterone sulfate, and androstenedione plasma levels. During the EE-CPA treatment, gonadotropins were suppressed, and the sex hormone-binding globulin level increased. Finasteride induced a decrease in dehydroepiandrosterone sulfate and 5α-dihydrotestosterone and an increase in testosterone levels.

Very few side-effects were observed during treatment with low doses of flutamide, EE-CPA, and particularly finasteride. Flutamide induced a decrease whereas EE-CPA induced an increase in triglycerides and cholesterol, showing higher values within the normal range. Ketoconazole induced several side-effects and complications, and several people dropped out of the study.

Despite different modalities of action and significantly different effects on androgen levels, low doses of flutamide, finasteride, and EE-CPA constitute very satisfactory alternative therapeutic regimens in the treatment of hirsutism. (*J Clin Endocrinol Metab* **84**: 1304–1310, 1999)

FLUTAMIDE (1–5), finasteride (6–10), ketoconazole (11–14), and cyproterone acetate (CPA) (15–20) are commonly employed in the treatment of hirsutism. Different therapeutic regimens have been used successfully; however, only a few randomized controlled trials exist, and subjective methods of evaluation are generally employed.

The aim of the present report was to compare, in a prospective, comparative, randomized study, as objectively as possible, the therapeutic efficacy as well as the endocrine and metabolic effects and reliability of low dose regimens of flutamide, finasteride, ketoconazole, and a combination of CPA and ethinyl estradiol (EE).

Subjects and Methods

Sixty-six premenopausal hirsute women (mean ± SD age, 22.9 ± 4.7) were referred to the Reproductive Medicine Unit of the University of Bologna (Bologna, Italy) for evaluation and treatment of hirsutism. The mean ± SD body weight was 61 ± 10 kg, and the mean ± SD height was 163 ± 6 cm. The mean body mass index (BMI) was 22.7 ± 2.7 (normal range, 18–24); 11 subjects (16%) were overweight (mean BMI, 27 ± 2.6).

Regular menses were reported by 29 of the 66 women; 32 had oligomenorrhea, 3 had amenorrhea, and 2 had polymenorrhea. Thirty-eight patients (58%) had ovulatory cycles (on the basis of typical progesterone levels in the premenstrual phase), and 28 (42%) had anovulatory cycles. Each patient underwent a complete medical and gynecological examination. In accordance with our codified parameters (21), all subjects had an etiological diagnosis of hirsutism. None of the women gave evidence of a hormonally active adrenal gland, an ovarian tumor, or Cushing's, PRL, or thyroid disorder. Twenty-seven patients (41%) had a diagnosis of polycystic ovary syndrome; 18 had anovulatory or oligoovulatory cycles, elevated plasma LH concentrations (LH/FSH ratio >2), high levels of testosterone and androstenedione, and ecographic evidence of enlarged polycystic ovaries. Nine patients had the concomitant presence of high dehydroepiandrosterone sulfate levels. Fourteen hirsute patients (21%) suffered from a mild form of nonclassic adrenal hyperplasia with high 17α-hydroxyprogesterone values, as diagnosed by ACTH test (21). Twenty-five patients (38%) were classified as having idiopathic hirsutism because they did not present any of the clinical features found in the other groups and had ovulatory cycles. In the entire population studied the hirsutism score ranged from 7–22.

Patients were randomized into four groups for treatment, indepen-

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dently of the diagnosis made. No significant differences were observed between groups regarding the prevalence of the diagnosis of hirsutism, clinical parameters, and menstrual rhythm or ovulatory/anovulatory cycles; thus, the patients were randomized into four comparable groups. Group 1 (n = 15; age, 22.6 ± 4 yr; BMI, 22.9 ± 2 kg/m^2) received 250 mg/day flutamide, group 2 (n = 15; age, 23.2 ± 3 yr; BMI, 22.5 ± 3 kg/m^2) received 5 mg/day finasteride, group 3 (n = 16; age, 23.2 ± 5 yr; BMI, 23.0 ± 3 kg/m^2) received 300 mg/day ketoconazole, and group 4 (n = 20; age, 22.9 ± 4 yr; BMI, 22.6 ± 2 kg/m^2) received a treatment regimen with low EE and CPA doses (the patients received 0.01 mg EE/day for the first week, 0.02 mg EE/day for the second week, and 0.01 mg EE/day for the third week, followed by a pause of 7 days, then 12.5 mg CPA/day administered in a reverse sequential regimen during the first 10 days of each treatment cycle). We planned a 12-month therapy period, and either the barrier method or intrauterine contraception was employed during the study in sexually active women to avoid any risk of conception.

Clinical and hormonal controls were performed in basal conditions and after 3, 6, 9, and 12 months. Each woman was studied during the early follicular phase of her menstrual cycle (3–6 days after the onset of a spontaneous menstrual flow) when present or at random in amenorrheic patients in basal conditions and after 180 and 360 days of treatment.

The study was approved by the ethical committee of the Institute of Obstetrics and Gynecology of the University of Bologna, and informed consent was obtained from each patient. All procedures followed in this study were in accordance with the Helsinki Declaration of 1975.

Hirsutism evaluation, clinical side-effects, endocrine and biochemical parameters [blood glucose, cholesterol, high density lipoprotein cholesterol, triglycerides, aspartate aminotransferase (AST), alanine aminotransferase (ALT), γ-glutamyltransferase, alkaline phosphatase, bilirubin, antithrombin III, and fibrinogen] were determined at each control visit.

Hirsutism and hair growth evaluation

Hirsutism was evaluated with a 2-fold criteria of control so as to have a method of analysis as objective as possible. 1) Hirsutism grading was codified by calculating the hirsutism score according to the modified Ferriman and Gallwey method (22), and the normal range was considered to be no higher than 8. Two patients with a total score of 7 were included because a severe regional (upper lip and thigh) hair growth, and the entry criteria for all patients was a score of 7 or greater. 2) As previously reported (12), hair parameters were codified using an IBAS image analyzer (Kontron Bildanalyze GmbH, Munich, Germany), a special image analysis processor with a sensitivity of 0.001 mm. First, all patients were shaved in a prefixed area of the right thigh. After 90 days, at least 20 hairs (basal hair growth) were cut from the same thigh area using curved scissors; then the area was reshaved, and treatments were started. This same procedure was repeated after 90, 180, 270, and 360 days of therapy. The mean diameter, hair length, and daily growth rate (obtained by dividing the length of each hair by the number of the days elapsed between shaving and the subsequent cut) were estimated. Considering that anagen hair growth on the thigh has a mean duration of 22 days and that the complete hair cycle is 84 days (23), a 90-day period of basal observation seems to be correct for establishing the mean basal hair growth in each group.

Self-reported evaluation

Patients' self-evaluation of the clinical outcome of the treatment was obtained. Each patient rated his appreciation as dissatisfied, satisfied, or highly satisfied.

Hormone assay

Blood samples were drawn at 0800 h; all samples from each subject were run in the same RIA. The RIA techniques used for hormonal measurements were: gonadotropins (FSH-LH) and PRL, rapid double antibody (kits purchased from Biodata, Rome, Italy); 17-hydroxyprogesterone (17-P) and progesterone (P), chromatographic separation on Sephadex LH-20 columns; dehydroepiandrosterone sulfate (DHAS) performed directly on diluted plasma, testosterone (T), 5α-dehydrotestosterone (DHT), androstenedione (A), and 17β-estradiol (E_2), TLC on silica gel 60 F254; dehydroepiandrosterone (DHA), plasma extraction with ethyl ether, as previously described (24), using an ACTH kit purchased from CIS (Gif-Sur-Yvete, France) and a cortisol (F) kit purchased from Diagnostic Systems Laboratories (Webster, TX); free testosterone (Tf), the Coat-A-Count free testosterone procedure of Diagnostic Products (Los Angeles, CA); and sex hormone-binding globulin (SHBG), noncompetitive liquid phase immunoradiometric assay (Farmos Diagnostic, Oulunsalo, Finland).

Statistical analysis

Paired and unpaired Student's *t* tests and ANOVA were used for statistical analysis, as needed. Values are expressed as the mean ± SD.

Results

Hirsutism

Figure 1 shows the results of the different regimens of therapy during the entire treatment period. In each subject who finished the study, not less than 100 hairs were analyzed by IBAS, both under basal conditions and during the entire cycle of treatment. Under basal conditions, 1848 hairs were analyzed, and 6800 hairs were analyzed during the entire cycle of treatment.

In group 1 (flutamide), hirsutism improved in all subjects. The mean basal score (Fig. 1; 14.2 ± 4.5) progressively decreased and dropped to 6.4 ± 3.5 ($P < 0.001$; −55 ± 13%) after 12 months of treatment. The mean diameter (Fig. 1) fell from 0.169 ± 0.02 to 0.133 ± 0.02 mm ($P < 0.001$; −21 ± 14%), and the mean daily rate of hair growth (Fig. 1) fell progressively from 0.153 ± 0.03 to 0.084 ± 0.04 mm/day ($P < 0.001$; −41 ± 18%).

In group 2 (finasteride), hirsutism improved in all subjects. The mean basal score (Fig. 1; 12.4 ± 4.8) slowly dropped during therapy to 6.9 ± 3.5 ($P < 0.02$; −44 ± 13%) after 12 months of treatment. The mean diameter (Fig. 1) fell from 0.174 ± 0.02 to 0.147 ± 0.02 mm ($P < 0.001$; −16 ± 12%), and the mean daily rate (Fig. 1) of hair growth fell from 0.127 ± 0.05 to 0.095 ± 0.04 mm/day ($P < 0.005$; −27 ± 14%).

In group 3 (ketoconazole), 8 of 16 subjects who started the therapy stopped taking the drug within 180 days because of several side-effects and complications; hirsutism had improved in the 8 subjects who concluded the 12-month therapy period (mean basal score, 13.8 ± 4.4; 12-month therapy score, 6.5 ± 4.8; $P < 0.005$; −53 ± 18%; Fig. 1). The mean diameter (Fig. 1) fell progressively from 0.177 ± 0.01 to 0.148 ± 0.02 mm ($P < 0.005$; −14 ± 12%), and the mean daily rate of hair growth (Fig. 1) fell from 0.129 ± 0.03 to 0.090 ± 0.04 mm/day ($P < 0.005$; −30 ± 21%). Six of 8 hirsute subjects who interrupted the therapy had slower hair growth during the therapy on the basis of their subjective evaluations.

In group 4 (EE-CPA), hirsutism improved in all subjects. The mean basal score (Fig. 1; 13.3 ± 5.1) dropped to 6.1 ± 5.5 ($P < 0.001$; −60 ± 18%) after 12 months of treatment. The mean diameter (Fig. 1) fell from 0.164 ± 0.05 to 0.138 ± 0.02 mm ($P < 0.001$; −20 ± 11%). The mean daily rate (Fig. 1) of hair growth decreased during the first 90 days and fell from 0.127 ± 0.05 to 0.090 ± 0.03 mm/day ($P < 0.001$) (−28 ± 21%) after 12 months.

Comparative data among groups

There were no significant differences among groups with respect to their clinical basic data, endocrine parameters

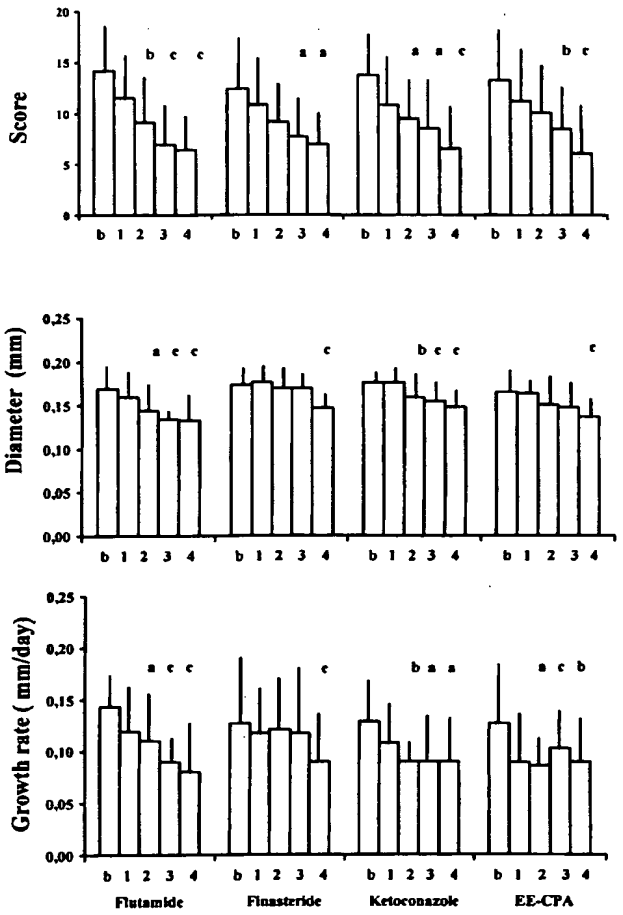

FIG. 1. Histograms representing the mean ± SD hirsutism score, diameter, and daily growth rate of hair under basal conditions (b) and after 90 (1), 180 (2), 270 (3), and 360 (4) days of therapy with different treatment regimens. In the ketoconazole group, 2 patients of 16 dropped out during the first 90 days of therapy, and 6 patients dropped out during the first 180 days. At 180, 270, and 360 days of treatment, only 8 subjects were studied. a, $P < 0.05$; b, $P < 0.01$; c, $P < 0.005$ (*vs.* basal within each group). For hirsutism score: by ANOVA among groups in basal conditions, P = NS; by ANOVA among groups during treatment, P = NS. For hair diameter: by ANOVA among groups in basal conditions, P = NS; by ANOVA among groups during treatment, significant differences at 90 ($F = 3.9$; $P < 0.01$), 180 ($F = 3.9$; $P < 0.01$), and 270 ($F = 6.7$; $P > 0.01$) days. For hair growth: by ANOVA among groups in basal conditions, P = NS; by ANOVA among groups during treatment, significant differences at 90 ($F = 3.6$; $P < 0.02$) and 180 ($F = 5.1$; $P < 0.003$) days.

(Tables 1 and 2), hirsutism score, or basal hair characteristics (Fig. 1).

For the hirsutism score (Fig. 1), no differences (by ANOVA, P = NS) were observed among the groups during the control period, confirming the efficacy of all four treatments. However significantly higher differences were observed in the percentage of decrease (Fig. 2) at the end of treatment in the cases of flutamide *vs.* finasteride ($-55 \pm 18\%$ *vs.* $-44 \pm 13\%$; $P < 0.05$) and EE-CPA *vs.* finasteride ($-60 \pm 18\%$ *vs.* $-44 \pm 13\%$; $P < 0.01$).

For hair diameter (Fig. 1), significant differences were observed at 90 days ($F = 3.94$; $P < 0.01$), 180 days ($F = 3.6$; $P < 0.01$), and 270 days ($F = 6.7$; $P < 0.001$) among the groups. Flutamide induced the quickest decrease in hair diameter during treatment (at 90 days of treatment) even though the differences in the percent decrease disappeared at the end of treatment (Fig. 2).

For the hair growth rate (Figs. 1 and 2), significant differences among groups were observed at 90 days ($F = 3.6$; $P < 0.02$) and 180 days ($F = 5.1$; $P < 0.003$). Finasteride was the slowest in decreasing hair growth, whereas EE-CPA was the quickest. Flutamide acted progressively, and there was a significant difference between flutamide and finasteride ($-41 \pm 18\%$ *vs.* $-27 \pm 14\%$; $P < 0.05$) at the end of treatment (Fig. 2).

Hormone concentrations

Tables 1 and 2 show the mean hormonal values under basal conditions and during the different treatments. In group 1, flutamide induced a significant decrease in 17-P, Tf, T, DHT, A, DHAS, DHA, and F and an increase in E_2 levels. In group 2, finasteride induced a significant decrease in DHAS, E_2, and DHT mean values and an increase in T and Tf mean values. In group 3, the mean plasma levels of T, Tf, DHT, DHA, DHAS, and A decreased progressively during ketoconazole treatment, whereas FSH, LH, E_2, F, ACTH, and 17-P values increased. In group 4 during treatment with EE/CPA, there was a decrease in LH, E_2, 17-P, T, Tf, A, DHA, and DHAS and an increase in F and SHBG plasma levels.

Side-effects, complications, and clinical and biochemical changes (Table 3)

In group 1, only a few, transient and slight side-effects occurred, and all subjects concluded the period of treatment. After 6 months of treatment, cholesterol (−11%) and triglycerides (−22%) values dropped significantly, with respect to the basal values. No changes were observed in the other parameters.

In group 2, no side-effects, complications, or biochemical changes were observed, even though two patients were dissatisfied.

In group 3, major side-effects and complications occurred during the first 90 days of treatment. A high number of patients dropped out within the first 180 days. Mean AST, ALT, and alkaline phosphatase levels increased progressively to the upper limit of the normal adult range. Two subjects had very high AST and ALT values. Triglycerides (−25%) and cholesterol (−17%) values decreased progressively. No changes were observed in the other parameters.

In group 4, some subjects experienced a mild weight gain (<2 kg) after 6–9 months of treatment and had mild transient side-effects. One subject suffered from irregular menstrual bleeding, and one dropped out because of persistent amenorrhea during the ninth month of therapy. Cholesterol mean values increased progressively (+21%) up to the upper limit of normal values (<250 mg/dL), and mean triglycerides

TABLE 1. Endocrine mean ± SD plasma values in basal conditions and after 180 and 360 days of therapy with different treatment regimens

	LH (IU/L; normal, 2–10)	FSH (IU/L; normal, 4–10)	PRL (μg/L; normal, 6–28)	E_2 (pmol/L; normal, 51.4–194.5)	ACTH (pmol/L; normal, 5–60)	17P (nmol/L; normal, 0.9–3.8)	SHBG (nmol/L; normal, 16–120)
Flutamide							
A	5.3 ± 2.6	5.5 ± 1.0	17 ± 6	154 ± 44	6.9 ± 3.1	3.3 ± 1.2	29 ± 11
B	4.8 ± 1.9	5.4 ± 0.8	18 ± 8	243 ± 36[a]	5.9 ± 2.9	2.8 ± 1.6	34 ± 11
C	6.0 ± 2.4	5.5 ± 1.0	18 ± 10	250 ± 33[a]	5.5 ± 2.7	2.2 ± 0.8[b]	37 ± 6
Finasteride							
A	6.0 ± 2.2	5.7 ± 1.6	15 ± 8	130 ± 37	6.1 ± 0.4	2.7 ± 0.9	38 ± 15
B	6.0 ± 2.1	6.0 ± 1.4	11 ± 5	130 ± 41	5.2 ± 0.5	2.2 ± 0.8	38 ± 12
C	5.1 ± 1.9	5.5 ± 1.2	13 ± 4	55 ± 31[a]	7 ± 0.4	2.7 ± 0.8	43 ± 16
Ketoconazole							
A	5.7 ± 2.8	4.2 ± 1.3	16 ± 6	180 ± 50	3 ± 2.1	3 ± 1.1	26 ± 10
B	10.2 ± 4.7[c]	5.4 ± 2.1	17.3 ± 5	280 ± 45[a]	7 ± 2[a]	5 ± 5.3	28 ± 11
C	12.5 ± 5.3[d]	6.8 ± 2.7[e]	17 ± 5	270 ± 33[a]	6 ± 1.9[d]	6 ± 3.2[c]	27 ± 10
EE-CPA							
A	4.7 ± 2.1	4.8 ± 1.2	12 ± 5	176 ± 52	5.4 ± 1.8	3.2 ± 1.1	30 ± 11.8
B	3.0 ± 1.6[b]	4.9 ± 2.0	18 ± 10	127 ± 62[e]	4.8 ± 1.6	2.2 ± 0.9[d]	124 ± 45[a]
C	3.2 ± 1.2[b]	4.7 ± 1.9	17 ± 10	92 ± 37[a]	4.4 ± 1.2	1.8 ± 0.6[a]	113 ± 38[a]

Normal hormonal ranges are reported in *parentheses*. In the ketoconazole group, at 180, 270, and 360 days of treatment, only eight subjects were studied. A, Basal; B, 180 days; C, 360 days. Significance is given for values within the same regimen.

[a] $P < 0.001$ *vs.* basal.
[b] $P < 0.05$ *vs.* basal.
[c] $P < 0.05$ *vs.* basal.
[d] $P < 0.005$ *vs.* basal.
[e] $P < 0.02$ *vs.* basal.

TABLE 2. Endocrine mean ± SD plasma values in basal conditions and during treatment after 180 and 360 days of therapy with four different treatment regimens

	T_f (pmol/L; normal, 2.4–12.5)	T (nmol/L; normal, 1–3.5)	DHT (nmol/L; normal, 0.3–1.1)	DHA (nmol/L; normal, 8.5–41.2)	DHAS (μmol/L; normal, 1.9–10.3)	A (nmol/L; normal, 1.4–15.7)	F (nmol/L; normal, 193–662)
Flutamide							
A	8 ± 3.1	1.7 ± 0.6	0.8 ± 0.2	40.8 ± 14	9 ± 2	10.8 ± 2.5	510 ± 57.9
B	8.7 ± 5.2	1.8 ± 0.7	0.7 ± 0.2	31 ± 10[a]	6 ± 1.8[b]	7.5 ± 2.9[c]	455 ± 49.6[d]
C	5.8 ± 2.7[a]	1.1 ± 0.2[b]	0.6 ± 0.1[c]	26.7 ± 10.7[c]	5.9 ± 1.8[b]	7.4 ± 1.6[a]	480 ± 55.1
Finasteride							
A	5.5 ± 2.4	1.7 ± 0.2	0.7 ± 0.2	37.7 ± 21.1	9 ± 2	7.9 ± 2.1	474 ± 63
B	6.5 ± 2.4	2.0 ± 0.2[b]	0.6 ± 0.2	38.1 ± 12.1	7 ± 1.9[d]	9 ± 2.3	469 ± 77
C	7.4 ± 2.4[a]	2.3 ± 0.2[b]	0.5 ± 0.1[c]	25.3 ± 12.9	7 ± 1.9[d]	9.2 ± 2.5	496 ± 74
Ketoconazol							
A	7 ± 2	1.6 ± 0.7	0.7 ± 0.3	32.1 ± 14.5	9 ± 2	7.5 ± 3	500 ± 200
B	5 ± 3	1.2 ± 0.2[b]	0.5 ± 0.2	20.5 ± 8[a]	7.5 ± 2.1	4.4 ± 3.1[a]	850 ± 197[b]
C	5 ± 2[a]	1.2 ± 0.1[b]	0.4 ± 0.1[c]	20.6 ± 7.5[a]	6.2 ± 1.9[c]	4.4 ± 2.7[a]	800 ± 255[d]
EE-CPA							
A	5.8 ± 2.4	1.8 ± 0.5	0.7 ± 0.3	35 ± 15.2	9 ± 1.8	9.1 ± 2.2	518 ± 102
B	4.1 ± 2.0[e]	1.1 ± 0.5[b]	0.8 ± 0.3	29.1 ± 15.4	7 ± 2[c]	7.1 ± 2.7[e]	596 ± 80[e]
C	3.8 ± 1.7[c]	1.3 ± 0.5[c]	0.8 ± 0.3	19.4 ± 6.5[b]	6 ± 2[b]	6.4 ± 1.8[b]	626 ± 55.1[b]

A, Basal; B, 180 days; C, 360 days. See Table 1 legend. Significance is given for values within the same regimen.

[a] $P < 0.05$ *vs.* basal.
[b] $P < 0.001$ *vs.* basal.
[c] $P < 0.005$ *vs.* basal.
[d] $P < 0.01$ *vs.* basal.
[e] $P < 0.02$ *vs.* basal.

values increased progressively (+38%), but they remained within the normal range (20–175 mg/dL).

Discussion

A very long treatment period is always required to improve hirsutism and prevent or delay its relapse; the use, as much as possible, of low doses of antiandrogens may be a suitable choice in an attempt to prevent the incidence of side-effects and complications and to maintain treatment. The present study confirms the effectiveness of all four antiandrogens, flutamide, finasteride, ketoconazole, and cyproterone acetate, in the treatment of hirsutism, even if given in very low doses; however, some differences do exist.

Low as well as high doses of flutamide alone (4, 25) were effective. The hirsutism score decreased progressively (−55%), and the improvement of hirsutism was associated with a rapid decrease in hair diameter (−11% after 3 months) and a progressive decrease in the daily hair growth rate (−41% after 12 months). A net decrease in T, Tf, DHT, A, 17-P, DHA, and DHAS plasma levels during treatment was observed, in agreement with some reports (26, 27) and in disagreement with others (1, 2, 5, 28), suggesting that the

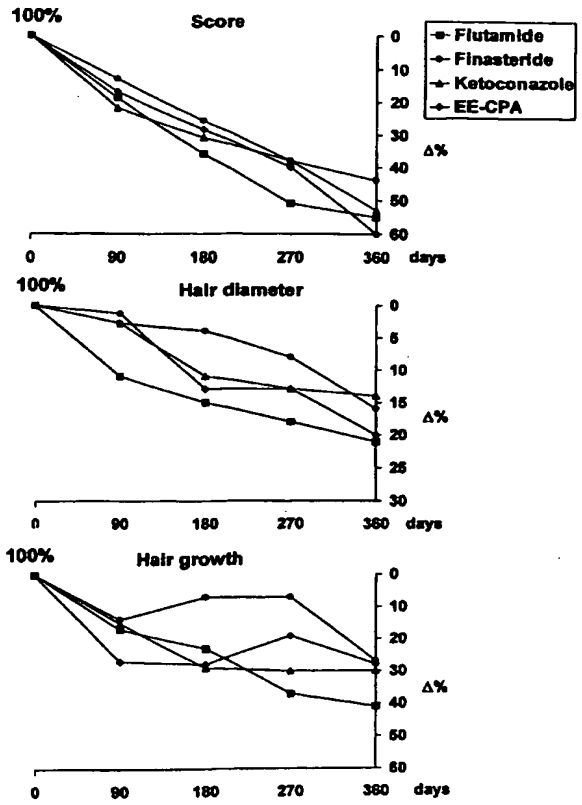

FIG. 2. Mean decreases in hirsutism score, hair diameter, and hair growth rate detected during the different treatments. Decreases are expressed as percentages with respect to basal values. For score: by ANOVA among groups during treatment, significant differences at 360 days ($F = 3.1$; $P < 0.03$); maximum change in the percent flutamide *vs.* finasteride after 360 days, $P < 0.05$; maximum change in the percent EE-CPA *vs.* finasteride after 360 days, $P < 0.01$. For hair diameter: by ANOVA among groups during treatment, significant differences at 90 ($F = 8.6$; $P < 0.001$) and 180 ($F = 3.1$; $P < 0.03$) days. For hair growth: by ANOVA among groups during treatment, significant differences at 90 ($F = 3.1$; $P < 0.03$), 180 ($F = 5.2$; $P < 0.003$), and 270 ($F = 9.9$; $P < 0.001$) days. For maximum change in percent flutamide *vs.* finasteride after 360 days, $P < 0.05$.

efficacy of flutamide must be ascribed to a reduction of androgen synthesis and to its action on target tissues. At a dose of 250 mg/day flutamide, neither liver failure nor the side-effects generally seen with high doses (2, 4, 27, 29, 30) were observed. Moreover, a significant decrease in the cholesterol and triglycerides values was observed, in contrast to the data reported by Dodin *et al.* (4).

Finasteride, generally used at a dose of 5 mg/day (6–10), caused a significant decrease in the hirsutism score (−44%), hair diameter (−16%), and daily hair growth rate (−27%). In our experience, the results were less satisfactory than those reported by some (7–10), but in accordance with others (31, 32). The administration of finasteride has been associated with a decrease in DHT plasma values (7, 8, 10) and an increase in T levels (7–10, 31); moreover, our data showed a decrease in DHAS levels, suggesting a slight inhibitory effect on adrenal steroidogenesis. Clinical parameters did not change, and no side-effects were reported during the administration of finasteride, confirming its favorable clinical applicability.

Using a low dose ketoconazole treatment regimen, the hirsutism score improved markedly in the patients who completed 12 months of therapy (−53%), and both hair diameter (−14%) and daily hair growth rate (−30%) decreased significantly. However, eight patients dropped out of the study because of side-effects and complications. We believe that ketoconazole should be used with caution. Our findings confirm a decrease in T, Tf, A, DHT, and DHAS values and an increase in 17-P values, and give evidence of a strong inhibition of 17- hydroxylase, 17,20-desmolase, and 11-hydroxylase activity (12, 33, 34).

High EE-CPA doses are very effective (15–18, 20); in the present study we used very low amounts of EE and CPA, and we confirm their high efficacy. Hirsutism progressively improved (as much as 60% less), and hair shaft diameter decreased significantly (−20%), even though the initial slowing of hair growth (−30% at 90 days) did not improve any further after the first 3 months of therapy. The beneficial effect of EE-CPA seems to be related to the well known peripheral effect and to the decrease in both ovarian and adrenal androgens as well as the increase in SHBG reached despite the low EE doses given. The effects of the EE-CPA on lipid metabolism are still being debated (35–38). Despite the low EE doses employed, an increase in triglycerides, which remained within the normal range, and a slight increase in total cholesterol were observed. The few transient side-effects reported during CPA-EE treatment did not require discontinuing the therapy.

In correctly evaluating and comparing the results of different treatments of hirsutism, we added the evaluation of the hair growth rate to the hirsutism score (22) and hair shaft diameter (31). This is a parameter whose behavior is partially independent of the hirsutism score and hair diameter (39), and this may explain the differences in the time response to the different therapies.

Finasteride appears to be the drug with the slowest time of action; it induces the least decrease in hirsutism score of the four treatments. However, the drug is highly effective, as the hair diameter at the end of the treatment is similar to that found with the other therapies. It is the best tolerated, and this therapy is probably the most effective in treating normoandrogenic hirsutism.

The EE-CPA combination induces the quickest reduction of hair growth and gives the greatest hair score decrease after 12 months of treatment. It is the treatment of choice in ovarian and adrenal hirsutism in sexually active women, because of steroid suppression, contraceptive effects (40) due to gonadotropin suppression, and optimal control of the menstrual cycle.

Flutamide has a quick and progressive effect on all parameters up to 12 months, very similar to the effects of EE-CPA and ketoconazole. The strong suppression of both adrenal and ovarian androgens without interference with gonadotropin secretion, the improved glucose tolerance (41), the cholesterol and triglycerides reduction, as well as the

TABLE 3. Side-effects, complications, and clinical changes during therapy with different treatment regimens

	Flutamide	Finasteride	Ketoconazole	EE-CPA
Headache	2[a]		3	2[a]
Nausea	1		4	
Astenia			4	
Wt gain[b]				5
Change of libido				1
Mastodinia[a]				2
Loss of scalp hair			4	
Menstrual irregularity			3	2
AST-ALT[c]			2	
Cholesterol (mg/dL)[d]				
A	185 ± 22		180 ± 32	180 ± 38
C	165 ± 29[e]	NS	150 ± 15[f]	227 ± 20[g]
Triglycerides (mg/dL)[d]				
A	63 ± 23		71 ± 18	65 ± 24
C	49 ± 12[e]	NS	53 ± 13[f]	106 ± 36[g]
Satisfied		3	4	
Highly satisfied	15	10	6	20
Dissatisfied		2	6	
Drop out			8	1

Values given are the number of subjects. A, Basal; C, 360 days. Significance is given for values within the same regimen.
[a] Slight and transient.
[b] Mild weight gain less than 2 kg.
[c] Aspartate amino-transferase-alanine amino-transferase.
[d] Mean values ± DS of all treated subjects.
[e] $P < 0.05$ *vs.* basal.
[f] $P < 0.01$ *vs.* basal.
[g] $P < 0.001$ *vs.* basal.

absence of side-effects at low doses enable this drug to be used in a flexible way, especially for hyperandrogenism in nonsexually active adolescents, in obese subjects, and in patients at cardiovascular risk.

Ketoconazole improves all parameters of hirsutism, as do the other treatments; however, in view of its important and frequent side-effects and complications, its use should be discouraged.

Finally, we emphasize that the treatment of hirsutism is aimed at the cause, and that each drug acts in its own way on anagen or telogen, hair diameter, or hair growth. However, the drugs employed in the present report together with spironolactone (42), which competes at the androgen receptor level, currently constitute very satisfactory alternative therapeutic regimens in the treatment of hirsutism.

References

1. **Muderris II, Bayram F, Sahin Y, Kelestimur F, Tutus A, Ayata D.** 1996 The efficacy of 250 mg/day flutamide in the treatment of patients with hirsutism. Fertil Steril. 66:220–222.
2. **Marcondes J, Minnani SL, Luthold WW, Wajchemberg BL, Samojlik E, Kirschner MA.** 1992 Treatment of hirsutism in women with flutamide. Fertil Steril. 57:543–547.
3. **Fruzzetti F, De Lorenzo D, Ricci C, Fioretti P.** 1993 Clinical and endocrine effects of flutamide in hyperandrogenic women. Fertil Steril. 60:806–813.
4. **Dodin S, Faure N, Cedrin I, et al.** 1995 Clinical efficacy and safety of low-doses of flutamide alone and combined with an oral contraceptive for the treatment of idiopathic hirsutism. Clin Endocrinol (Oxf). 43:575–582.
5. **Couziner B, Pholsena M, Young J, Schaison G.** 1993 The impact of a pure antiandrogen (flutamide) on LH, FSH, androgens and clinical status in idiopathic hirsutism. Clin Endocrinol (Oxf). 39:157–162.
6. **Tolino A, Petrone A, Sarnacchiaro F, et al.** 1996 Finasteride in the treatment of hirsutism: new therapeutic perspectives. Fertil Steril. 66:61–65.
7. **Moghetti P, Castello R, Magnani C, et al.** 1994 Clinical and hormonal effects of the 5α-reductase inhibitor finasteride in idiopathic hirsutism. J Clin Endocrinol Metab. 79:1115–1121.
8. **Ciotta L, Cianci A, Calogero AE, Palumbo MA, Marletta E, Sciuto A, Palumbo G.** 1995 Clinical and endocrine effects of Finasteride, a 5α-reductase inhibitor, in women with idiopathic hirsutism. Fertil Steril. 64:299–306.
9. **Castello R, Tosi F, Perrone F, Negri C, Muggeo M, Moghetti P.** 1996 Outcome of long-term treatment with 5α-reductase inhibitor finasteride in idiopathic hirsutism: clinical and hormonal effects during a 1-year course of therapy and 1-year follow-up. Fertil Steril. 66:734–740.
10. **Fruzzetti F, De Lorenzo D, Parrini D, Ricci C.** 1994 Effects of finasteride, a 5α-reductase inhibitor, on circulating androgens and gonadotropin secretion in hirsute women. J Clin Endocrinol Metab. 79:831–835.
11. **Martikainen H, Heikkinen J, Ruokonen A, Kauppila A.** 1988 Hormonal and clinical effects of ketoconazole in hirsute women. J Clin Endocrinol Metab. 66:987–991.
12. **Venturoli S, Fabbri R, Dal Prato R, et al.** 1990 Ketoconazole therapy for women with acne and/or hirsutism. J Clin Endocrinol Metab. 71:335–339.
13. **Gal M, Orly J, Barr, I, Algur N, Boldes R, Diamant YZ.** 1994 Low-dose ketoconazole attenuates serum androgen levels in patients with polycystic ovary syndrome and inhibits ovarian steroidogenesis *in vitro*. Fertil Steril. 61:823–832.
14. **Vidal-Puig AJ, Munos-Torres M, Joder-Gimeno E, et al.** 1994 Ketoconazole therapy: hormonal and clinical effects non-tumor hyperandrogenism. Eur J Endocrinol. 130:333–338.
15. **Hammerstein J, Meckies J, Leo-Rosserg I, Moltz L, Zielske F.** 1975 Use of cyproterone acetate (CPA) in treatment of acne, hirsutism and virilism. J Steroid Biochem. 6:827–836.
16. **Underhill R, Dewhurst J.** 1979 Further clinical experience in the treatment of hirsutism with cyproterone acetate. Br J Obstet Gynecol. 86:139–141.
17. **Belisle S, Love EJ.** 1986 Clinical efficacy and safety of cyproterone acetate in severe hirsutism: results of a multicentered Canadian study. Fertil Steril. 46:1015–1020.
18. **Flamigni C, Venturoli S, Fabbri R.** 1988 Long-term management of hirsute patients with cyproterone acetate. In: Spona J, Aydinlik S, eds. Hirsutism and endocrine dermatological problems. Carnforth: The Parthenon Publishing Group; 29–37.
19. **McKenna TJ.** 1991 The use of the anti-androgens in the treatment of hirsutism. Clin Endocrinol (Oxf). 35:1–3.
20. **Barth JH, Cherry CA, Wojnarowska F, Dawber RPR.** 1991 Cyproterone acetate for severe hirsutism: results of a double-blind dose-ranging study. Clin Endocrinol (Oxf). 35:5–10.
21. **Venturoli S, Fabbri R, Paradisi R, Gammi L, Jasonni VM, Flamigni C.** 1980 Diagnostica degli irsutismi: test dinamici surrenalici. Giorn It Ost Gin. 2/4:15–23.
22. **Hatch R, Rosenfield R, Kim MH, Treway D.** 1981 Hirsutism: implications, etiology, and management. Am J Obstet Gynecol. 140:815–830.
23. **Seago SV, Ebling FJG.** 1985 The hair cycle on the human thigh and upper arm. Br J Dermatol. 113:9–16.
24. **Paradisi R, Venturoli S, Pasquali R, et al.** 1986 Effects of obesity on gonadotropin secretion in patients with polycystic ovarian disease. J Endocrinol Invest. 9:139–144.
25. **Muderris II, Bayram F, Sahin Y, Kelestimur F.** 1997 A comparison between

two doses of flutamide (250 mg/d and 500 mg/d) in the treatment of hirsutism. Fertil Steril. 68:644–647.
26. **Cusan L, Dupont A, Gomez JL, Tremblay RR, Labrie F.** 1994 Comparison of flutamide and spironolactone in the treatment of hirsutism: a randomized controlled trial. Fertil Steril. 61:281–287.
27. **Moghetti P, Castello R, Negri, et al.** 1995 Flutamide in the treatment of hirsutism: long-term clinical effects, endocrine changes, and androgen receptor behavior. Fertil Steril. 64:511–517.
28. **Motta T, Maggi G, Perra M, Azzolari E, Casazza S, D'Alberton A.** 1991 Flutamide in the treatment of hirsutism. Int J Gynecol Obstet. 36:155–157.
29. **Ciotta L, Cianci A, Marletta E, Pisana L, Agliano A, Palumbo G.** 1994 Treatment of hirsutism with flutamide and a low dosage oral contraceptive in polycystic ovarian disease patients. Fertil Steril. 62:1129–1135.
30. **Wilow I, Fourcroy JL.** 1996 Flutamide hepatotoxicity. J Urol. 155:209–212.
31. **Wong IL, Morris RS, Chang L, Spahn M, Stanczyc FZ, Lobo R.** 1995 A prospective randomized trial comparing finasteride to spironolactone in the treatment of hirsute women. J Clin Endocronol Metab. 80:233–238.
32. **Erenus M, Yucelten D, Durmusuglu F, Gurbuz O.** 1997 Comparison of finasteride *vs.* spironolactone in the treatment of idiopathic hirsutism. Fertil Steril. 68:1000–1003.
33. **Couch RM, Muller J, Perry YS, Winter JSD.** 1987 Kinetic analysis of human adrenal steroidogenesis by oral ketoconazole: J Clin Endocrinol Metab. 65:551–555.
34. **Rajfer J, Sikka SC, River F, Handelsman DJ.** 1986 Mechanism of inhibition of human testicular steroidogenesis by oral ketoconazole. J Clin Endocrinol Metab. 63:1193–1198.
35. **Wynn V, Godsland F, Seed M, Jacobs S.** 1986 Paradoxical effects of the anti-androgen cyproterone acetate on lipid and lipoprotein metabolism. Clin Endocrinol (Oxf). 24:183–191.
36. **Falsetti L, Pasinetti E.** 1995 Effects of long-term administration of an oral contraceptive containing ethinylestradiol and cyproterone acetate on lipid metabolism in women with polycystic ovary syndrome. Acta Obstet Gynecol Scand. 74:56–60.
37. **Gaspard UJ.** 1988 Endocrine and metabolic effects of low dose combination of ethinylestradiol and cyproterone acetate in hirsute women. In: Spona J, Aydinlik S, eds. Hirsutism and endocrine dermatological problems. Carnforth: Parthenon Publishing Group; 47–60.
38. **Porcile A, Gallardo E.** 1991 Long-term treatment of hirsutism: desogestrel compared with cyproterone acetate in oral contraceptives. Fertil Steril. 55:877–881.
39. **Barth JH.** 1997 Semi-quantitative measurements of body hair in hirsute women compare well with direct diameter measurements or hair shafts. Acta Derm Venereol. 77:317–318.
40. **Venturoli S, Ravaioli B, Bagnoli A, et al.** 1998 Contraceptive and therapeutic effectiveness of two low-dose ethinylestradiol and cyproterone acetate regimens in the treatment of hirsute patients. Eur J Contrac Reprod Health Care. 2:1–5.
41. **Moghetti P, Tosi F, Castello R, et al.** 1996 The insulin resistance in women with hyperandrogenism is partially reversed by antiandrogen treatment: evidence that androgens impair insulin action in women. J Clin Endocrinol Metab. 81:952–960.
42. **Schapiro G, Evron S.** 1988 A novel use of spironolactone: treatment of hirsutism. J Clin Endocrinol Metab. 51:429–432.

sson J. A. and Kaptein R.: he glucocorticoid receptor ience 249 (1990) 157–160.
D. B., French F. S., Jewell A.: Antibodies to steroid acid binding domains and man glucocorticoid receptor. 018–1026.
R., Lombes M., Nahon E., ger B. F.: Identification of DNA-binding domain of nd the B chain of insulin. 8K 2142.

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INTERACTION OF GLUCOCORTICOID ANALOGUES WITH THE HUMAN GLUCOCORTICOID RECEPTOR

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Summary—Transient co-transfection of receptor cDNA and suitable reporter genes was used to study human glucocorticoid receptor (hGR) function in a neutral mammalian cell background. A variety of natural and synthetic steroids were analyzed for their ability to activate gene expression through the hGR and to bind to extracts of cells expressing the hGR cDNA. There was very good correlation between these two *in vitro* parameters for these compounds. Furthermore, correlation of these data with reported *in vivo* anti-inflammatory potencies was surprisingly close, with two exceptions. The *in vitro* data suggest an explanation for the discrepant compounds, consistent with published data on their metabolic fate *in vivo*. The co-transfection assay has utility as a quantitative predictor of *in vivo* glucocorticoid pharmacology.

INTRODUCTION

The human glucocorticoid receptor (hGR), cloned in 1985 [1], is known to be a member of a protein superfamily of closely related intracellular receptors (IRs) which function as ligand-activated transcription factors [2–4]. The hormone–IR complex can positively or negatively regulate the expression of gene networks by its interaction with specific target hormone response elements (HREs) within the promoters of controlled genes. One useful development in the elucidation of the structure and function of the GR protein was the development of a "*cis–trans*" or co-transfection assay in which glucocorticoid-dependent transcriptional control could be reconstituted in a model cell system [5]. This has enabled significant advances in the understanding of the domain structure of the hGR and other IRs [5–7]. In the "*cis–trans*" assay, a plasmid encoding the cDNA for the hGR under a constitutive promoter, e.g. the Rous sarcoma virus (RSV) long terminal repeat (LTR), and a second plasmid carrying a gene for a detectable reporter, e.g. firefly luciferase (LUC), under the control of a glucocorticoid-responsive promoter, e.g. the LTR of the mouse mammary tumor virus (MMTV), are introduced into a neutral mammalian cell background. This results in reconstitution of hormone-dependent transcriptional transactivation of reporter gene expression. This introduction of the hGR cDNA and the MMTV LTR-LUC can be accomplished by preparing suitable adenoviral vectors [9] or by transient transfection of two plasmids, one directing overexpression of hGR and the other encoding MMTV LTR-LUC. In addition to conferring on recipient CV-1 cells a functional and measurable response to glucocorticoids, adenoviral infection or transient transfection with the hGR-encoding vector also confers specific binding of tritiated dexamethasone.

The availability of a reliable and quantitative *in vitro* predictor of *in vivo* anti-inflammatory activity would have significant benefit. In the present study, we evaluate the utility of *in vitro* quantitative assessment of various glucocorticoid analogues using the "*cis–trans*" assay and radioligand binding as predictors of *in vivo* activity of these compounds. The potency and efficacy of a panel of 21 natural and synthetic glucocorticoids were assessed. The functional agonist activity of these 21 glucocorticoids was determined in CV-1 cells expressing transfected hGR cDNA over a full range of concentrations (10^{-11} to 10^{-5} M). This was compared to their potency to displace specifically bound tritiated dexamethasone from cytoplasmic extracts of CV-1 cells expressing the hGR protein after introduction of hGR cDNA.

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EXPERIMENTAL

Media and chemicals

CV-1 cells were grown as a monolayer in Dulbecco's modified Eagle's medium (DMEM) containing 10% (v/v) fetal calf serum (Gibco, Grand Island, NY) and supplemented with 2 mM L-glutamine (Gibco), and 55 μg/ml gentamicin (Flow Laboratories, McLean, VA). Plasmids pRShGR, pGRE-LUC, and pRSV-β-gal have been previously described [5]. Briefly, pRShGR is a pBR322 derivative containing the hGR cDNA under control of the RSV LTR. pGRE–LUC contains a cDNA for LUC under the control of the MMTV LTR, a conditional promoter containing a glucocorticoid response element (GRE). pRSV-β-gal contains the gene for *E. coli* β-galactosidase under control of the RSV-LTR, a constitutive promoter. Unlabeled chemicals were obtained from Sigma Chemical Co. (St Louis, MO). [^{3}H]Dexamethasone (approx. 40 Ci/mmol) was purchased from Amersham Radiochemicals (Arlington Heights, IL).

Buffers and enzyme assays

For the competitive binding assay, homogenization buffer [(10 mM Tris–HCl (pH 7.4) 1 mM EDTA, 1 mM phenylmethylsulfonylfluoride, 2 mM dithiothreitol, 0.25 M sucrose)] and gelatin phosphate buffer [0.15 M NaCl, 0.015 M NaN_3, 0.1 M Na_2HPO_4, 0.039 M NaH_2PO_4 (pH 7.0), 0.1% gelatin] were utilized.

Buffers utilized for the calcium phosphate mediated co-transfection assay were as described [8]. All cell washing steps were in 150 mM phosphate buffered saline (PBS). Test ligands were prepared in DMEM containing 10% (v/v) charcoal-absorbed fetal calf serum. Luciferase activity in cell extracts was assayed in 9.7 mM $MgCl_2$, 1.66 mM ATP, 0.45 sodium luciferin, 91 mM potassium phosphate, pH 7.8 on a Dynatech luminometer as described [9]. Cell extracts were also analyzed for β-galactosidase activity as described previously [5].

Preparation of cell extracts

CV-1 cells were infected with Ad/MLUC7 [9], a recombinant adenovirus expressing the hGR cDNA, in the presence of dl309 helper virus (5 PFU/cell) by incubating cells and viruses in DMEM with 10% FBS (DMEM/FBS) at 37°C. After a 30 min adsorption, the mixture was diluted 10-fold with DMEM/FBS and plated in 15 cm culture dishes. At the end of an 18 h incubation at 37°C, cells were detached and washed with PBS by centrifugation at 700 g for 5 min. All further procedures were carried out at 4°C. Cells were homogenized in 2 vol of homogenization buffer in a Teflon homogenizer with a motor-driven pestle at 1250 rpm. The homogenate was clarified by centrifugation at 1000 g for 15 min. A soluble cytosol fraction was generated by centrifuging this supernatant fraction at 104,000 g for 1 h. The protein concentration of the cytosol fraction was determined by dye binding [10], using bovine γ-globulin as standard. Cytosol fractions were used immediately or stored at −70°C.

Competitive binding assay

Aliquots of cytosol (100 μg protein) were incubated at 4°C with 2.5 nM tritiated dexamethasone in the presence of incremental concentrations (0–2.5 × 10^{-5} M) of unlabeled dexamethasone or unlabeled cold competitor test compounds. After a 24 h incubation period at 4°C, unbound steroid was removed by addition of 2 vol of 7.5% dextran-coated charcoal in gelatin-phosphate buffer. The mixture was incubated for 10 min at 4°C and centrifuged at 3000 g for 10 min. The radioactivity in the supernatant fluid was determined by liquid scintillation counting. The non-specific binding was determined in the presence of unlabeled dexamethasone (10^{-5} M). All determinations were performed in duplicate.

Co-transfection assay

Co-transfections were performed essentially as described [5]. Sub-confluent CV-1 cells were passed at 3 day intervals to maintain good transfection efficiency. CV-1 cells were plated 24 h prior to transfection at 70% confluency. The recombinant DNA constructs were transiently transfected into CV-1 cells by calcium-phosphate co-precipitation [8]. Each plasmid preparation used for transfection was cesium banded twice prior to use. Following transfection, all subsequent steps were performed on the Biomek Beckman Automated Workstation. Medium was removed from transfected cells after 6 h, cells were washed and each glucocorticoid was tested at seven incremental concentrations in duplicate (10^{-11}–10^{-5} M). After 38 h the cells were washed and lysed with 0.5% Triton-X 100 and assayed for luciferase and β-galactosidase activities, using a luminometer (Dynatech) and ELISA plate reader. The EC_{50} (concentration giving 50% of maximal observed

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RESULTS

For each compound tested, a full concentration–response curve was determined in the co-transfection assay, using the hGR cDNA and an MMTV-LUC reporter in CV-1 cells as described (Experimental). Representative data are shown in Fig. 1(A). In this assay, in the absence of added compound, the basal LUC activity is essentially undetectable (< 1 relative light unit, RLU). After exposure to fully efficacious glucocorticoids, e.g. dexamethasone, greater than 600 RLUs are obtained. If a control plasmid is substituted for that containing the hGR cDNA, fully efficacious concentrations of analogues give fewer than 5 RLUs (data not shown). Testing of the solvent at concentrations used to dissolve test substances had no effect on LUC activity (data not shown). When hGR is introduced, the concentration–response curve saturates, giving the maximal response above fully active concentrations. The transition from no measurable response to full response occurs over approximately two logs of concentration. Most active analogues give approximately full efficacy, although the partial efficacy displayed by fluocinolone [approx. 40%, Fig. 1(A)] was reproducible; fluocinolone produces no transactivation of MMTV-LUC in CV-1 in the absence of introduced hGR cDNA (data not shown). For each analogue, an EC_{50} was determined graphically as the concentration giving 50% of maximal effect for that compound. The EC_{50} data are compiled in Table 1; both the absolute EC_{50} (M) and a value normalized to that of hydrocortisone are given. Fluocinolone is the most potent compound (EC_{50} = 150 pM), being about 200-fold more potent than hydrocortisone. The least potent compound tested with detectable activity was progesterone (EC_{50} = 2.5 μM), almost 80-fold less potent than hydrocortisone.

Extracts of CV-1 cells infected with adenovirus engineered to encode the hGR cDNA were prepared as described (Experimental) and used to analyze the ability of the various steroids to displace 2.5 nM [^{3}H]dexamethasone. Representative data are shown in Fig. 1(B). Non-specific binding was < 2% of specific binding under these assay conditions [9]. The maximal binding was > 20-fold for extracts of cells infected with Ad/MLUC7 compared to mock-infected cell extracts [9]. For compounds capable of competing with [^{3}H]dexamethasone, including fluocinolone, > 95% of the specific

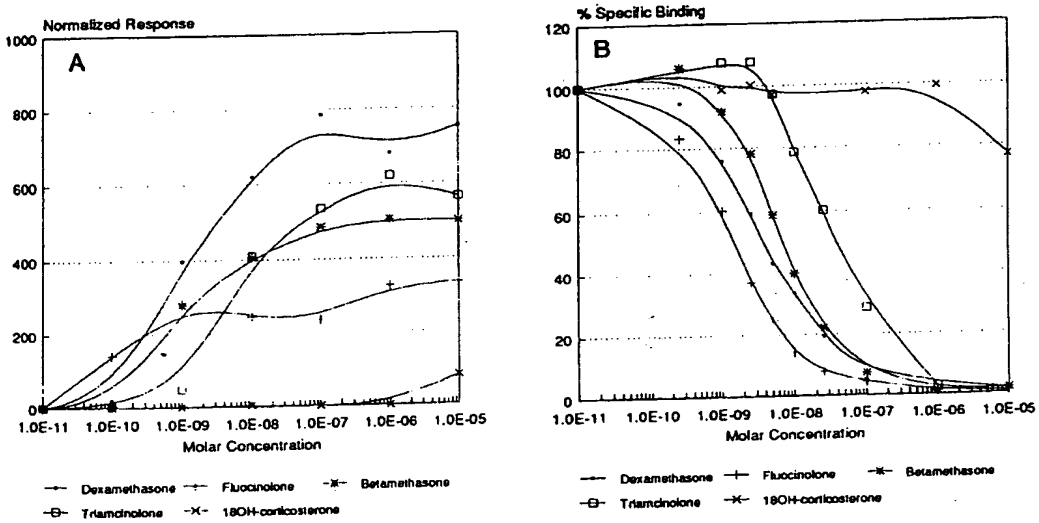

Fig. 1. Concentration dependence of transactivation and radioligand displacement by selected glucocorticoid analogues. Representative *in vitro* data are shown for several of the glucorticoid analogues tested. *Panel A*: dependence of hGR-mediated activation of the LUC reporter gene (ordinate) on concentration of glucocorticoid analogue in the culture medium (abscissa); and *Panel B*: concentration dependence of competitive displacement of 2.5 nM [^{3}H]dexamethasone from over-expressed hGR by several synthetic glucocorticoid analogues, expressed as percent of control binding (ordinate) as a function of added unlabeled competitor compound (abscissa).

Table 1. Transactivation and binding of glucocorticoid analogues to hGR

Compound	Co-transfection EC_{50}	Co-transfection Normalized	Binding IC_{50}	Binding Normalized
Dexamethasone	1.2E-09	0.04	5.8E-09	0.12
Fluocinolone	1.5E-10	0.00	1.5E-09	0.03
Betamethasone	8.1E-10	0.03	7.0E-09	0.14
Triamcinolone	5.3E-09	0.17	4.0E-08	0.80
6α-Methyl prednisolone	1.3E-08	0.41	9.0E-09	0.18
Fludrocortisone	1.4E-08	0.44	1.3E-08	0.26
Prednisolone	2.7E-08	0.84	1.6E-08	0.32
Hydrocortisone	3.2E-08	1.00	5.0E-08	1.00
Corticosterone	4.7E-08	1.47	1.0E-07	2.00
Aldosterone	7.5E-08	2.34	7.2E-07	14.40
21-Deoxycortisol	2.5E-07	7.81	1.2E-07	2.40
11-Deoxycorticosterone	5.9E-07	18.44	5.0E-08	1.00
11-Deoxycortisol	7.6E-07	23.75	1.5E-07	3.00
Progesterone	2.5E-06	78.13	5.0E-08	1.00
Prednisone	> 1.0E-05		2.0E-06	40.00
Cortisone	> 1.0E-05		3.8E-06	76.00
Testosterone	> 1.0E-05		5.5E-06	110.00
Dihydrotestosterone	—		1.0E-05	200.00
Estriol	> 1.0E-05		> 1.0E-05	
Tetrahydrocortisone	> 1.0E-05		> 1.0E-05	
Tetrahydrocortisol	> 1.0E-05		> 1.0E-05	
Tetrahydrocorticosterone	> 1.0E-05		> 1.0E-05	
18-Hydroxydeoxycorticosterone	> 1.0E-05			
18-Hydroxycorticosterone	> 1.0E-05		> 1.0E-05	

Note: normalization to hydrocortisone.

binding was displaced over approximately a two log concentration range. Representative data are shown in Fig. 1(B). Table 1 gives graphically determined concentrations required to inhibit 50% of specific binding of 2.5 nM [^{3}H]dexamethasone (IC_{50}), both as absolute values (M) and normalized to the IC_{50} of hydrocortisone. IC_{50} values range over three logs, with fluocinolone the most potent compound and testosterone the least.

A comparison of normalized potency in the co-transfection assay and in the competitive

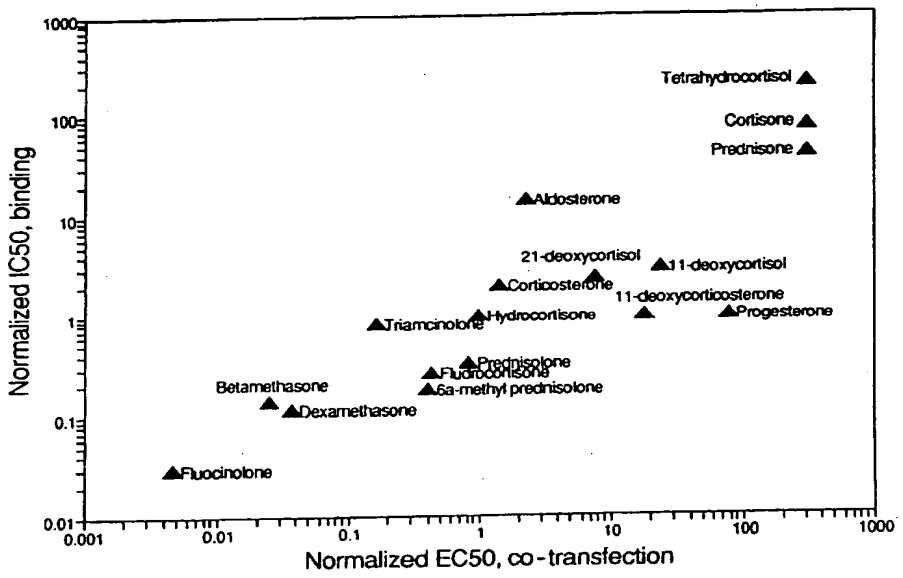

Fig. 2. Comparison of steroid binding and transactivation. Relative binding potency (ordinate) vs relative transactivation potency (abscissa). Competitive binding potency was determined for each analogue by measuring IC_{50}, i.e. the concentration required to inhibit by 50% specific binding of 2.5 nM [^{3}H]dexamethasone to extracts of CV-1 cells over-expressing hGR cDNA as described (Experimental). Relative binding potency was derived by normalizing these data to the IC_{50} of hydrocortisone. Transactivation potency was measured for each analogue in the co-transfection assay using hGR cDNA in CV-1 cells as described (Experimental). Data are expressed as EC_{50}, normalized to the EC_{50} of hydrocortisone.

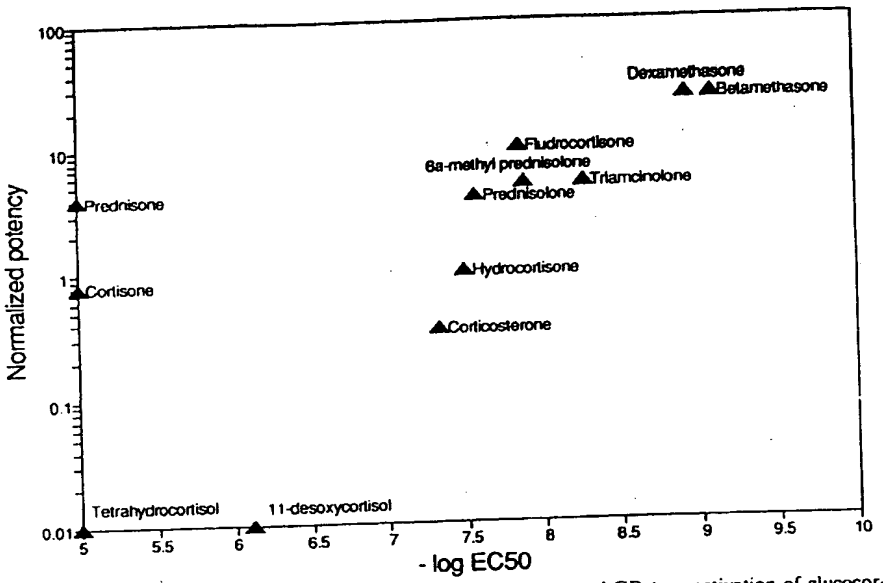

Fig. 3. Comparison of *in vivo* anti-inflammatory potency and *in vitro* hGR transactivation of glucocorticoid analogues. Relative anti-inflammatory potency *in vivo* (ordinate) vs *in vitro* transactivation potency (abscissa). Relative anti-inflammatory potency, normalized to that of hydrocarbons, is derived from reported values [11]. Transactivation potency was determined in the *in vitro* co-transfection assay using hGR cDNA introduced into CV-1 cells as described (Experimental). Values are expressed as—log EC_{50}.

binding assay for the various analogues (Fig. 2) reveals an excellent correlation between these two parameters. A notable exception to this correlation is progesterone, which is equipotent with hydrocortisone in displacing [^{3}H]dexamethasone, but about 80-fold weaker as a functional agonist of the hGR in the co-transfection assay.

Comparison of the biological potencies of the various steroids analyzed in the co-transfection assay *in vitro* compared to reported values of their *in vivo* anti-inflammatory potency ([11] based on dose in milligrams necessary for equal efficacy) is presented graphically in Fig. 3. Very good agreement is noted for all compounds, with the exception of prednisone and cortisone, which are much more potent *in vivo* than they are in the *in vitro* co-transfection assay in CV-1 cells.

DISCUSSION

The comparison of binding potency and transactivation agonist activity *in vitro* (Table 1 and Fig. 2) shows good correlation between the two for most of the 21 compounds examined. The partial agonist activity observed with fluocinolone (Fig. 1) does not appear to result from interaction of the compound with a subset of the introduced hGR, since fluocinolone gives no agonist activity in CV-1 cells in the absence of transfected hGR cDNA and is capable of displacing 100% of the specifically bound [^{3}H]dexamethasone from hGR-expressing CV-1 cell extracts. It is possible that the conformation of the fluocinolone–hGR complex is less effective than full agonists at interacting with other components of the transcriptional apparatus in CV-1 cells.

The present study underscores the feasibility of using the "*cis–trans*" assay for quantitative evaluation of potential hGR agonist. There are several significant theoretical advantages of the "*cis–trans*" assay over conventional radioligand binding assays. The most significant is that the assay determines not only whether a compound interacts with hGR but also the functional consequences of that interaction on gene expression, allowing the prediction of agonist and antagonist pharmacological effects. The assay, using hGR, can be expected to be less susceptible to potential species-related artifacts and inaccurate predictions than small-animal-based pharmacological studies. Finally, in screening for novel pharmacophores which might act as agonists or antagonists of the hGR, the "*cis–trans*" assay can detect any small molecules with functional consequences,

whether or not they interact with the receptor in the natural hormone binding site. Competitive radioligand displacement assays can only detect such compounds if their binding results in allosteric effects on the ligand site.

The agreement between the relative potencies of the 21 analogues as activators of hGR-dependent transcription and as competitors with dexamethasone binding was remarkably close (Table 1 and Fig. 2). There were a few compounds which showed greater ability to displace dexamethasone binding than ability to transactivate MMTV-LUC in CV-1 cells. Progesterone was equipotent with hydrocortisone in binding, but 80-fold less potent in transactivation. These data suggest that progesterone binds to the hGR but doesn't lead to the allosteric changes in hGR conformation necessary for transactivation. This failure could be at the level of dissociation of hGR and heat shock protein or at the level of interaction with the GRE. The data suggest that progesterone might antagonize glucocorticoid activation of the hGR.

In the case of the compounds for which estimates of *in vivo* anti-inflammatory potency were available, there was remarkably good correlation with the *in vitro* results in the "*cis–trans*" assay (Fig. 3). This reflects the extent to which the model cell system mirrors systemic sites of *in vivo* action. Two notable exceptions to this were prednisone and cortisone, both of which had low activity in the CV-1 cell assay. Formally, this could reflect either catabolism to inactive derivatives by CV-1 cells or the absence of necessary metabolic activation *in vitro*. The inactive compounds both have carbonyl functions at position 11. In the "*cis–trans*" assay, their 11-hydroxylated analogues, prednisolone and corticosterone, were about two logs more potent. Prednisone and cortisone were also relatively ineffective in displacing [^{3}H]dexamethasone from hGR-containing extracts at 4°C *in vitro*, arguing against catabolism as the explanation of their lack of potency in the "*cis–trans*" assay. Prednisone and cortisone are known to require hepatic metabolic activation to prednisolone and corticosterone for *in vivo* activity [11]. This activating metabolic conversion apparently does not occur in CV-1 cells *in vitro*. With the exception of these compounds requiring metabolic activation, the "*cis–trans*" assay is surprisingly predictive of *in vivo* potency.

In the present study, close correlation was found in most cases between the relative potencies of the 21 compounds in functional agonist activity *in vitro* and in radioligand displacement. Furthermore, there was excellent correlation between *in vitro* data and reported *in vivo* anti-inflammatory data for these compounds. Introduction by infection or co-transfection of hGR cDNA and a suitable reporter into a receptor-deficient mammalian cell establishes a hormone-inducible transcription system which be utilized to quantitate the pharmacological efficacy and potency of potential ligands for the human glucocorticoid receptor.

REFERENCES

1. Hollenberg S. M., Weinberger C., Ong E. S., Cereli G., Oro A., Lebo R., Thompson E. B., Rosenfeld M. G. and Evans R. M.: Primary structure and expression of a functional human glucocorticoid receptor cDNA. *Nature* **318** (6047) (1985) 635–641.
2. Evans R. M.: The steroid and thyroid hormone receptor superfamily. *Science* **240** (1988) 889–895.
3. Green S. and Chambon P.: Nuclear receptors enhance our understanding of transcription regulation. *TIG* **4** (11) (1988) 309–314.
4. Beato M.: Transcription control by nuclear receptors. *FASEB J.* **5** (1991) 2044–2051.
5. Giguere V., Hollenberg S. M., Rosenfeld M. G. and Evans R. M.: Functional domains of the human glucocorticoid receptor. *Cell* **46** (1986) 645–652.
6. Green S. and Chambon P.: Oestradiol induction of a glucocorticoid responsive gene by a chimaeric receptor. *Nature* **325** (1987) 75–78.
7. Godowski P. J., Rsuconi S., Miesfeld R. and Yamamoto K. R.: Glucocorticoid receptor mutants that are constitutive activators of transcriptional enhancement. *Nature* **325** (1987) 365–368.
8. Wigler M., Sweet R., Sim G. K., Wold B., Pellicer A., Lacy E., Maniatis T., Silverstein S. and Axel R.: Transformation of mammalian cells with genes from prokaryotes and eukaryotes. *Cell* **16** (1979) 777–785.
9. Shih W., Mears T., Bradley D. J., Parandoosh Z. and Weinberger C.: An adenoviral vector system for functional identification of nuclear receptor ligands. *Molec. Endocr.* **5** (2) (1991) 300–309.
10. Bradford M. M.: A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principal of protein-dye binding. *Analyt. Biochem.* **72** (1976) 248–254.
11. Haynes R. C. Jr and Murad F.: Adrenocorticotropic hormone; adrenocortical steroids and their synthetic analogues; inhibitors of adrenocortical steroid biosynthesis In *The Pharmacological Basis of Therapeutics* (Edited by A. G. Gilman, L. S. Goodman, T. W. Rall and F. Murad). MacMillan Publishing Co., New York (1985) pp. 1459–1489.

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